REMARKS

In response to the Office Action mailed August 9, 2007, reconsideration is respectfully requested in view of the above amendments and the following remarks. Applicants have amended claims 91 and 94 and new claims 127 and 128 have been added, support for which may be found throughout the specification as originally filed. The above amendments are not to be construed as acquiescence to the Examiner's stated grounds for rejection and are made without prejudice to prosecution of any subject matter removed or modified by this amendment in a related divisional, continuation or continuation-in-part application.

Claims 91, 94, 99, 105-107, 111, 117-119 and 121 remain rejected under 35 U.S.C. § 103 as allegedly obvious over various combinations of previously cited references, including Johnson *et al.*, Kensil *et al.*, De Vries *et al.*, and Mossman *et al.* The basis for these maintained rejections appears to be predicated on the Examiner's belief that the references provide motivation to combine AGPs and saponins generally, and that Applicants have failed to provide evidence of unexpected or synergistic results that can be extrapolated across the full scope of the claimed invention.

Applicants respectfully traverse. By the above amendment, for purposes of clarity and in a good faith effort to advance prosecution of this application, Applicants have amended claims 91 and 94 to specify that the recited saponin is a saponin selected from QS-21 and QS-7, or a combination thereof. Consequently, the invention as presently claimed is drawn to methods involving selection from a narrow class of AGP compounds and from the two specifically recited saponins, QS-21 and QS-7.

Johnson *et al.* (U.S. Patent No. 6,113,918) describes AGP compounds but does not teach or suggest combining an AGP compound with QS-21 or QS-7, much less that the specific combination as claimed by Applicants would give rise to a synergistic immune response.

Mossman *et al.* (WO02/03961) describes a nucleic acid delivery system using microspheres. Mossman *et al.* also describes that AGPs and/or other adjuvants may be used in conjunction with the described microspheres. Mossman *et al.* does not, however, teach or suggest combining any specific AGP compound with QS-21 and/or QS-7, or that by selecting a combination as claimed by Applicants a synergistic immune response would result.

Johnson *et al.* (US2001/0053363) describes isotucerasol-based saponin mimetic compounds that are structurally distinct from QS-21 and QS-7, and their use as adjuvants. Johnson *et al.* describes, in Example 7, that isotucerosol was combined with an AGP compound and that this combination enhanced serum antibody production (Example 7, paragraph 0207). Johnson *et al.* also describes, in Example 7, that this combination has an additive effect with respect to a CTL-based immune response (Example 7, paragraph 0208).

Thus, the cited references, which, according to the Examiner provide the motivation to combine Applicants' claimed AGPs and saponins, clearly fail to teach the specific combinations claimed by Applicants, much less the synergistic properties achieved when such combinations are selected and used. The fact that two general classes of compounds may have been previously described does not render specific combinations obvious when those specific combinations possess unexpected properties that were not derivable from the prior art. Indeed, the references cited by the Examiner offer nothing of substance that would lead the skilled artisan to any expectation that synergistic immune responses could be achieved by selection of the combinations as presently claimed, and Johnson *et al.* in fact teaches away from any such expectation. As Applicants' claimed combinations give rise to synergistic results that would not have been expected and could not have been predicted in view of the disclosures of the prior art, the claimed invention is submitted to be non-obvious over these references.

Regarding the Examiner's assertion that there is no rationale that the synergistic effects as presented in the declaration of August 24, 2006, would be extrapolated to all the Quil A fractions herein and to all the AGPs encompassed by the claims, the evidence presented in the prior Declarations is indeed reasonably commensurate in scope with evidence of unexpected results for the invention as claimed. The Declaration demonstrated that three structurally distinct AGPs within the narrow scope of AGPs claimed gave rise to synergistic results when used in combination with QS-21. Further, as specifically stated in the prior Declaration, these results were believed to be predictive of the same or similar synergistic effects for other combinations encompassed within the scope of the claims.

As noted above, claims 91 and 94 have been limited to the saponins QS-21 and QS-7, and a skilled artisan would reasonably expect, based upon shared structural and functional

characteristics between these two saponins, that the synergistic results presented for the three AGP compounds in combination with QS-21 could be reasonably extrapolated to QS-7 as well. In further support of this position, Applicants have ***enclosed herewith*** the Declaration of Dr. Charlotte Kensil, a noted expert in this field.

Regarding the Examiner's statement that there is no evidence that the synergistic effect demonstrated by Applicants is unique and unexpected compared to other combinations taught or suggested by the prior art, it is not Applicants' burden to prove that all other possible combinations of adjuvants are not synergistic in order to establish that the claimed combinations are non-obvious. Applicants have demonstrated synergistic effects for multiple combinations within the scope of the current claims. Further, as there is no general expectation in the field of immunology that synergistic effects will be achieved when two given adjuvants are combined, Applicants' demonstration of synergy in the context of the claimed invention must be properly viewed as an unexpected result.

Finally, regarding the Examiner's statement that it is not clear how and why the asserted synergistic effects are practically significant, Applicants submit that a skilled artisan would not find it difficult to appreciate why synergistic effects would be preferred over additive effects when employing a combination of adjuvants. Adjuvants are used to enhance the immunogenicity of an antigen and a higher level of immunogenicity would clearly be viewed as preferred over a lower level of immunogenicity.

The Director is authorized to charge any additional fees due by way of this Amendment, or credit any overpayment, to our Deposit Account No. 19-1090.

Respectfully submitted,

SEED Intellectual Property Law Group PLLC

/Jeffrey Hundley/
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Enclosure:
Declaration of Dr. Charlotte Kensil Under 37 C.F.R. § 1.132

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Sally Mossman *et al.*
Application No.	:	10/068,171
Filed	:	February 4, 2002
For	:	IMMUNOSTIMULANT COMPOSITIONS COMPRISING AMINOALKYL GLUCOSAMINIDE PHOSPHATES AND SAPONINS
Examiner	:	Shengjun Wang
Art Unit	:	1617
Docket No.	:	210121.721
Date	:	

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. CHARLOTTE KENSIL UNDER 37 C.F.R. § 1.132

I, Charlotte Kensil, Ph.D., do hereby declare that:

1. I received a Ph.D. from the University of California, San Diego, in 1981. A copy of my CV outlining my experience in the field of saponin adjuvants is attached.

2. I have reviewed the prior Declarations submitted December 8, 2005, and August 24, 2006, and the data presented therein demonstrating that a synergistic immune response was elicited when QS-21 was combined with the three different AGP compounds RC-527, RC-540 and RC-557.

3. I have also reviewed the Examiner's position in the Office Action dated August 9, 2007, that there is no rationale that the synergistic effects as presented in the declaration of August 24, 2006, could be extrapolated to all of the saponins and AGPs encompassed by the pending claims.

4. I understand that the saponins recited in the claims as currently amended are limited to QS-21 and QS-7.

5. QS-21 and QS-7 share significant structural and functional similarities, which, in my opinion, lead to a reasonable expectation that QS-7, like QS-21, would give rise to a synergistic immune response when used in combination with the claimed AGPs.

6. Certain of the structural features shared between QS-21 and QS-7 include the following:

- the triterpene is identical in both compounds (quillaic acid);
- both compounds have a critical functional group (triterpene aldehyde) which appears essential for enhancing immune responses based on QS-21 structure/function studies (*e.g.*, Exhibit A, Soltysik et al, Figures 3 & 4);
- the trisaccharide attached to the 3-carbon of the triterpene is identical in both compounds, including the one charged group (glucuronic acid);
- the tetrasaccharide is the same in both compounds, with the exception that QS-7 has two extra terminal monosaccharides attached to the QS-21 tetrasaccharide (t-rhamnose, attached to the 3-hydroxyl of fucose, which is unsubstituted in QS-21 and t-glucose, attached to the 3-hydroxyl of rhamnose, which is unsubstituted in QS-21); and
- both compounds are acylated at the 4-position of the tetrasaccharide fucose.

7. In addition, certain of the functional similarities between QS-21 and QS-7 are noted below:

- both compounds induce CTL to subunit antigens (*e.g.*, Exhibit B, Kensil *et al.*, 1998, Figures 2 & 3);
- both compounds induce similar antibody responses to BSA and cytochromeb5 (*e.g.*, Exhibit C, Kensil *et al.*, 1991, Figures 2 & 3);
- the compounds induce very similar CTL responses when compared with the lower responses induced by MPL or IL-12 (Exhibit D, Slide A);
- both compounds are capable of inducing similar levels of CTL to OVA in C57BL/6 mice (Exhibit D, Slide B);
- both compounds are capable of enhancing interferon-gamma production after vaccination and restimulation with antigen (Exhibit D, Slide C);
- both compounds are capable of enhancing IgG1 and IgG2a antibody to OVA (Exhibit D, Slides D & E);

- both compounds enhance serum IgG and nasal IgA when administered intranasally with flu vaccine (Exhibit D, Slides F & G); and
- both compounds are capable of inducing natural killer cell responses, indicating that both influence innate immune responses (Exhibit D, Slide H).

8. In light of the structural and functional similarities between QS-21 and QS-7, it is my opinion that the synergistic immune responses previously demonstrated for combinations comprising QS-21 and the AGP compounds RC-527, RC-540 and RC-557 can be reasonably extrapolated to the other claimed saponin, QS-7.

9. It is also my opinion, based on the synergistic immune responses demonstrated using the three distinct AGP compounds RC-527, RC-540 and RC-557, in combination with QS-21, that the observed synergy can be reasonably extrapolated to other AGP compounds claimed.

11. I further hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true; and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-identified patent application and/or any patent issuing therefrom.

Charlotte Kensil
Signature of Charlotte Kensil, Ph.D.

February 8, 2008
Date of Signature

Enclosures:

Exhibit A : Soltysik *et al.* (Vaccine, Vol. 13, No. 15, 1403-1410, 1995)

Exhibit B: Kensil *et al.* (J. Immunology 146, 431-437, No. 2, 1991)

Exhibit C: Kensil *et al.* (Modulation of the Immune Response to Vaccine Antigens, Brown F. Haaheim LR (Eds) Dev Biol Stand Basel, Karger, 1998, vol 92, 41-47)

Exhibit D: Powerpoint Slides A-H

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Institution and Location	Degree Awarded	Year	Scientific Field
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University of California San Diego, California	Ph.D	1981	Chemistry

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Date	Position
6/81-12/85	Postdoctoral fellow. Biochemistry Department, University of Connecticut Health Center, Farmington, CT.
1/86-1/88	Staff Scientist, Protein Chemistry Department, Cambridge BioScience Corporation, 365 Plantation St., Worcester 01605.
1/88-8/93	Section Manager of Natural Products Chemistry Department, Biopharm Division, Cambridge Biotech Corporation, 365 Plantation St., Worcester, MA 01605.
8/93-10/96	Senior Director of Adjuvant and Drug Delivery Research, Cambridge Biotech Corporation, 365 Plantation St., Worcester, MA 01605
10/96-11/98	Senior Director of Adjuvant and Drug Delivery Research, Aquila Biopharmaceuticals Inc, 365 Plantation St., Worcester, MA 01605
11/98-11/00	Vice-president of Research, Adjuvants, Aquila Biopharmaceuticals Inc, 175 Crossing Boulevard, Framingham, MA 01702
11/00-3/03	Vice-president of Research, Antigenics Inc, 175 Crossing Boulevard, Framingham, MA 01702
3/03-present	Consultant, Vaccine Research

RESEARCH PUBLICATIONS:

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U.S. PATENTS:

1. Saponin Adjuvant.
   Inventors: **Charlotte A. Kensil** and Dante J. Marciani
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number: 5,057,540
   Issued: Oct. 15, 1991

2. Methods for Enhancing Drug Delivery with Modified Saponins
   Inventors: **Charlotte A. Kensil**, Sean Soltysik, and Dante J. Marciani
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number: 5,273,965
   Issued: Dec. 28, 1993

3. Vaccine Comprising Recombinant Feline Leukemia Antigen and Saponin Adjuvant
   Inventors: Gerald Beltz, Dante J. Marciani, C.-H. Hung, and **Charlotte A. Kensil**
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number: 5,352,449
   Issued: Oct. 4, 1994

4. Modified Saponins for Enhancing Drug Delivery
   Inventors: **Charlotte A. Kensil**, Sean Soltysik, and Dante J. Marciani
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number: 5,443,829
   Issued: Aug. 22, 1995

5. Saponin-Antigen Conjugates and the Use There of
   Inventors: **Charlotte A. Kensil,** Sean Soltysik, and Dante J. Marciani
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number 5,583,112
   Issued: Dec. 10, 1996

6. Drug Delivery Enhancement via Modified Saponin
   Inventors: **Charlotte A. Kensil**, Sean Soltysik, Dante J. Marciani, and Joanne Recchia
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number 5,650,398
   Issued: July 22, 1997

7. Saponin Adjuvant Composition
   Inventor: **Charlotte A. Kensil**
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number 6,231,859 B1
   Issued: May 15, 2001

8. Saponin Compositions and Uses Thereof
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   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number 6,524,584 B2
   Issued: February 25, 2003

9. Compositions of Saponin Adjuvants and Excipients
   Inventors: **Charlotte A. Kensil** and Gerald A. Beltz
   Assignee: Antigenics, Inc., Lexington, MA
   Patent Number 6,645,495 B1
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Structure/function studies of QS-21 adjuvant: assessment of triterpene aldehyde and glucuronic acid roles in adjuvant function

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QS-21, a purified Quillaja saponaria *saponin immunologic adjuvant, contains two functional groups that we hypothesized to be involved in the adjuvant mechanism of action through charge or Schiff base interaction with a cellular target. Derivatives, prepared by modification of these sites, were prepared and tested for their ability to augment the immunogenicity of the antigen ovalbumin (OVA) in C57BL/6 mice. QS-21 derivatives that were modified at the carboxyl group on an anionic sugar, glucuronic acid, retained adjuvant activity for antibody stimulation, inducing relative increases in antibody titers similar to those induced by QS-21, although the minimum adjuvant dose required for this stimulation was increased several fold relative to the dose of unmodified QS-21. One of these derivatives also retained significant activity for induction of OVA-specific cytotoxic T-lymphocytes. In contrast, QS-21 derivatives modified at an aldehyde on the triterpene did not show adjuvant activity for antibody stimulation or for induction of cytotoxic T-lymphocytes, suggesting that this functional group may be involved in the adjuvant mechanism.*

Keywords: QS-21; adjuvant; *Quillaja saponaria*; saponin; structure/function

Extracts of the bark of the South American tree *Quillaja saponaria* Molina contain a heterogeneous saponin fraction with potent adjuvant activity[1,2]. These saponins have been purified to near homogeneity by HPLC and characterized for adjuvant activity[3]. Several were shown to stimulate high antigen-specific antibody titers in mice[3,4]. One of these saponins, QS-21, was also shown to induce class I MHC-restricted cytotoxic T-lymphocyte (CTL) responses in mice when used with subunit antigens such as ovalbumin and recombinant HIV-1 envelope antigens[5,6] and to increase antibody titers to T-independent polysaccharide antigens[7]. QS-21 has also been tested as an adjuvant in a Phase I melanoma vaccine clinical trial and was noted to augment antigen-specific IgG titers[8], making it of particular interest as a vaccine adjuvant. However, relatively little is known of the minimum critical structure of QS-21 required for these adjuvant functions. This study addresses the relationship of QS-21 structure to its adjuvant function via analysis of two functional groups.

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QS-21 is a highly complex triterpene glycoside (*Figure 1*), with branched sugar chains at carbon 3 and carbon 28 on the triterpene quillaic acid[4]. A correlation between the presence of branched sugar chains at these positions and adjuvant activity of naturally occurring saponins was noted[9]. The glycoside on carbon 3 contains an anionic sugar residue, glucuronic acid, which imparts an overall negative charge to the QS-21 molecule at physiological pH. There is also an aldehyde on carbon 4 of the triterpene. In addition, QS-21 contains a fatty acid (3,5-dihydroxy-6-methyl-octanoic acid) linked through an ester bond to the 3-hydroxyl or 4-hydroxyl of fucose (N. Jacobsen, personal communication). An identical fatty acid is linked in ester bond to the 5-hydroxyl of the first fatty acid; the 5-hydroxyl of the second fatty acid is glycosylated with a single sugar (arabinose).

Relatively little is known of the minimum critical structure of QS-21 required for adjuvant function. There are several adjuvant active saponins that have been isolated from *Quillaja saponaria* Molina[3]. These include the saponins QS-7, QS-17, QS-18, and QS-21 which are the predominant saponins in the bark and which as an aggregate represent approximately half of the saponins present in *Quillaja saponaria* bark. Structural comparison suggests that the known adjuvant active saponins have the triterpene backbone (quillaic acid) and some

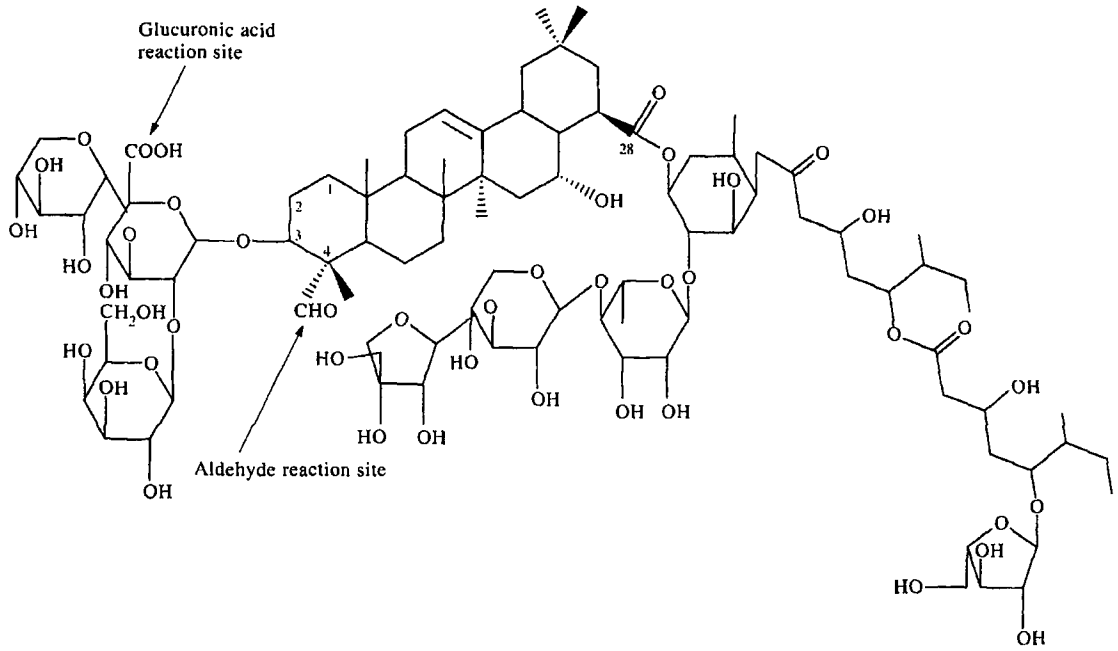

Figure 1 Structure of QS-21. The two functional groups selected for modification are shown

carbohydrate residues in common. Two structural features held in common by all adjuvant active saponins from *Quillaja saponaria* Molina are the 2,3 glucuronic acid[3,4,10] and the quillaic acid backbone, including the aldehyde at carbon 4[11]. We postulated that these functional groups were involved in the QS-21 adjuvant mechanism, the glucuronic acid through charge interaction and the aldehyde via Schiff base formation with a cellular target. Hence, these two functional groups (glucuronic acid carboxyl group and triterpene aldehyde) were modified by conjugation to small blocking groups; the resulting derivatives were tested as adjuvants in an effort to evaluate the importance of these functional groups or nearby regions to adjuvant function.

MATERIALS AND METHODS

Materials

QS-21 was purified from an aqueous extract of *Quillaja saponaria* bark by adsorption chromatography and reversed-phase HPLC[3]. N-hydroxysulfosuccinimide (S-NHS) and N,N'-dicyclohexylcarbodiimide (DCC) were purchased from Pierce Chemical Co. (Rockford, IL). Sodium cyanoborohydride, anhydrous dimethylformamide (DMF), anhydrous dimethylsulfoxide (DMSO), and 8-anilino-1-naphthalene-sulfonic acid (ANS) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Ovalbumin (OVA), Grade VI, was obtained from Sigma Chemical Co. (St. Louis, MO).

Preparation of derivatives modified at glucuronic acid carboxyl

Conjugates (1:1 mol:mol) of QS-21 and the small molecules ethylamine, glycine, and ethylenediamine were prepared. In order to react the glucuronic acid carboxyl group of QS-21 with free amino groups on the molecules used as blocking agents, an active ester derivative of QS-21 was prepared using sulfo-NHS[12]. A twofold molar excess of S-NHS and a threefold molar excess of DCC were added to 50 mM QS-21 in anhydrous DMF; this reaction mixture was incubated with stirring overnight at room temperature to generate the S-NHS active ester derivative of QS-21 (QS-21/S-NHS ester). The reaction mixture was then chilled on ice and filtered to remove dicyclohexylurea. The active ester derivative of QS-21 was then precipitated by addition of ethylacetate (final ratio=6/1 ethylacetate/DMF, v/v), purified by repeated washes with ethylacetate, collected by centrifugation (15 min at 1000*g*), and dried by vacuum desiccation overnight.

In order to conjugate the small molecules glycine, ethylamine, and ethylenediamine through their free amino groups to the glucuronic acid carboxyl of QS-21, a modification of the method of Anjaneyulu and Staros[13] was employed. A 100-fold molar excess of glycine, ethylamine, or ethylenediamine (1.0 M in 0.1 M sodium phosphate, pH 7.0) was added to solid QS-21/S-NHS active ester. The reaction mixture was stirred at room temperature for 1 h. The resulting conjugates were purified on a Vydac C4 column (1.0 cm I.D. × 25 cm length, 5 μm particle size, 300 angstrom pore size) using a Waters 600E HPLC system and detection at 214 nm (LambdaMax Model 481 Variable Wavelength Detector). A linear water/acetonitrile gradient in 0.15% trifluoroacetic acid was used. Pooled fractions containing the predominant reaction product were lyophilized to dryness.

Preparation of derivatives modified at triterpene aldehyde

To conjugate glycine, ethylamine, and ethylenediamine through their free amino groups to the triterpene aldehyde on QS-21 (*Figure 1*), a 50-fold molar excess of these compounds in 0.1 M sodium phosphate, pH 6.0

was added to QS-21 (12 mM) in 0.1 M sodium phosphate (pH 6.0)/methanol (50/50, v/v) and incubated with stirring overnight at room temperature to induce Schiff base formation. These adducts were stabilized by the use of sodium cyanoborohydride as a selective reducing agent[14]. Sodium cyanoborohydride (from a 0.1 M stock solution in methanol) was added to a final ratio of 4/1 (mol/mol) over QS-21. The reaction mixture was stirred overnight. The predominant reaction product was purified by HPLC as described above.

Characterization of QS-21 derivatives

Derivatives were characterized for purity and retention time relative to QS-21 by reversed-phase HPLC on C18 (3 μm particle size, 120 angstrom pore size, 4.6 mm I.D. × 15.0 cm length (YMC Inc., Wilmington, N.C.)) using a linear gradient of 80% solvent A/20% solvent B to 40% solvent A/60% solvent B over 20 min at a 1 ml min^{-1} flow rate. Solvent A was 0.1% H_3PO_4 in water and solvent B was 0.1% H_3PO_4 in acetonitrile. Detection was by UV absorbance at 205 nm. Relative retention time was determined from the ratio of k'derivative/k'QS-21 where k'=(peak retention time − void retention time)/(void retention time). Molecular weights were determined by fast atom bombardment-mass spectrometry (M-Scan Corp., Westchester, PA) to confirm that these derivatives were 1:1 covalent conjugates. ^{1}H-NMR on samples in deuterated dimethylsulfoxide was carried out by Spectral Data Services (Champaign, IL). Modification of the triterpene aldehyde in compounds (5)–(7) was confirmed by elimination of the aldehyde proton resonance (singlet with chemical shift =9.47 in unmodified QS-21). The aldehyde proton resonance was present in compound (3) whereas a proton singlet at 8.1 ppm (assigned as the proton resonance on the amide bond nitrogen) appeared. Compounds (4) and (5) were not assayed by NMR.

Immunizations

C57BL/6 mice (female, 8–10 weeks of age) were used for all immunizations. Mice were immunized subcutaneously with 0.2 ml of 25 μg OVA and varying amounts of QS-21 derivatives with either two or three immunizations spaced 2–3 weeks apart. Sera were collected one week after the second immunization or two weeks after the third immunization for analysis by EIA. Splenic mononuclear cells for use as the source of effector cells in the CTL assay were collected two weeks after the last of three immunizations.

Immunological assays

The EIA was done using OVA-coated Immulon IV plates (Dynatech Laboratories, Chantilly, VA). Plates were coated with OVA by overcoating wells with 100 μl per well of 10 μg ml^{-1} OVA in PBS and incubation at 4°C overnight. Plates were emptied and were then incubated for 1 h at ambient temperature with 150 μl $well^{-1}$ of 10% normal goat serum (Gibco Laboratories, Grand Island, NY) in PBS. Plates were washed three times with 0.05% Tween-20 in water. Serial dilutions of sera in 10% normal goat serum in PBS (1/10 dilutions) were prepared and incubated on the plate for 1 h at room temperature. Plates were then washed three times with 0.05% Tween-20 in water. For measurement of total IgG, a total volume of 100 μl goat anti-mouse IgG-horseradish peroxidase conjugate (BioRad, Richmond, VA), diluted 1/ 12 500 in 10% normal goat serum in PBS, was incubated on the plate for 1 h at room temperature. For measurement of IgG1, $IgG2_b$, or $IgG2_a$, a volume of 100 μl goat-antimouse IgG1, $IgG2_b$, or $IgG2_a$ (conjugated to alkaline phosphatase, Southern Biotechnology, Birmingham, AL, diluted 1/250 in 10% normal goat serum/PBS) was added to each well and incubated at 4°C overnight. Plates were washed five times with 0.05% Tween-20 in water followed by one wash with water. Color development was with tetramethylbenzidine substrate for total IgG and with *p*-nitrophenylphosphate for IgG1, $IgG2_b$, and $IgG2_a$.

CTL activity was assayed using splenic mononuclear cells as the source of CTL. The precursor CTL in splenic mononuclear cell preparations were induced to mature *in vitro* by culture with mitomycin C-treated E.G7-OVA cells at a 20:1 (responder:stimulator) ratio or with denatured ovalbumin. For a negative antigen control, splenic mononuclear cells were cultured with medium (supplemented RPMI 1640). Culture in presence or absence of antigen was carried out using supplemented RPMI 1640 medium at 37°C in a 2 ml volume with 1×10^6 cells per ml in culture tubes. Cells were recovered after 144 h of culture and used in the CTL assay. The CTL activity was measured using both EL4 cells and E.G7-OVA (EL4 cells transfected with cDNA coding for OVA[15]) as targets. Cytotoxicity was measured after 4 h of incubation of CTL with 10^4 ^{51}Cr-labeled target cells per well, using effector/target (E/T) ratios of 25:1 to 3:1. The percent of specific ^{51}Cr release was calculated as 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release) where maximum release was measured after lysis of target cells with 1% NP-40 and spontaneous release was measured after incubation of target cells with medium.

Hemolysis and critical micellar concentration assays

Hemolytic activity of QS-21 and derivatives were measured in an *in vitro* assay on sheep red blood cells. Five milliliters of sheep red blood cells in Alsever's solution (Biowhittaker, Walkersville, Maryland) were spun at 900*g* for 5 min. The pellet was resuspended in 5 ml PBS. This process was repeated twice. The final pellet was resuspended in 3 ml PBS. One hundred microliters of serial 1:2 dilutions of QS-21 or derivatives in PBS were added to individual wells of Falcon flexible round bottom 96 well assay plates (Becton Dickinson, Oxnard, CA). Twenty-five microliters of the washed red blood cell suspension were added to each well, mixed with the saponin solution, and incubated at room temperature for 30 min. The round bottom plate was then centrifuged at 1000*g* for 5 min. Fifty microliters of each of the supernatants were transferred to wells in a flat bottom microtiter plate for determination of the absorbance at 570 nm.

Critical micellar concentration (cmc) of QS-21 and derivatives was determined by a fluorescent dye binding assay as described previously[16,17]. The fluorescent probe ANS, 11 μM in phosphate-buffered saline, was mixed with different concentrations of QS-21 or derivative.

Immediately after mixing, fluorescence emission at 490 nm with excitation at 370 nm was determined. The fluorescence emission was plotted versus QS-21 or derivative concentration. Biphasic curves were obtained with low fluorescence emission below the cmc and significant increase in fluorescence emission above the cmc due to intercalation of ANS into the micelle. Best fit lines were determined for the biphasic curve; the cmc was defined as the QS-21 or derivative concentration corresponding to the intersection of the best fit lines.

RESULTS

Modification of QS-21

In order to evaluate the importance of the glucuronic acid and triterpene aldehyde to the adjuvant activity of QS-21, derivatives consisting of 1:1 conjugates of QS-21 linked to the small blocking groups glycine, ethylamine, and ethylenediamine at these functional groups were prepared. The size, charge, and hydrophobicity of these derivatives compared to QS-21 are summarized in *Table 1*. Although the size increase to QS-21 due to conjugation of these blocking groups was minimal, all of the conjugations were expected to sterically hinder or block any direct interactions at the modified sites. Some of these modifications also modified QS-21 charge, enabling an evaluation of whether the anionic carboxyl group on QS-21 was involved in a charge interaction as part of the adjuvant mechanism. In compound (3), the glucuronic acid was blocked with a neutral group (ethylamine), eliminating the charge at physiological pH. In compound (4), conjugation of the acid to ethylaminediamine resulted in a conjugate with a free amino group, imparting a cationic charge. Although modifications of the aldehyde did not affect the anionic group on the glucuronic acid carboxyl, this modification did affect the overall charge of the QS-21 molecule. Compound (6) is zwitterionic, with negative charges on the glucuronic acid and a positive charge on the secondary amine formed on the aldehyde, whereas compound (7) is positively charged overall. Hydrophobicity changes (assessed by relative retention time to QS-21 on reversed-phase HPLC) were determined to be minimal and were within the range encompassed by naturally occurring adjuvant-active saponins such as QS-7 (relative retention time 0.72).

Antibody stimulation

All derivatives were tested for activity in stimulating antigen-specific antibody to OVA in C57BL/6 mice. Mice received three immunizations with OVA and 10 μg of QS-21 (1) or derivatives (2)–(4) (modified at the glucuronic acid carboxyl group) or (5)–(7) (modified at the aldehyde at triterpene carbon 4). Anti-OVA IgG1, $IgG2_b$, and IgG1 titers were determined by EIA (*Figure 2*). Unmodified QS-21 induced significant increases in IgG1, $IgG2_b$, and $IgG2_a$. Derivatives (2) and (3) also induced significant increases in antibody of all three isotypes although to a slightly lesser extent than QS-21. No adjuvant effect was noted for derivative (4) except for a 10-fold increase in $IgG2_b$. The antibody profile induced by OVA formulations adjuvanted with derivatives (5)–(7) was similar to that induced by the OVA/

Table 1 Characterization of QS-21 derivatives

Derivative	Blocking group	Reaction site	Theoretical molecular formula (formula weight)[a]	m/z of pseudo-molecular ions (relative intensity)[b]	Assignment of FAB-MS ion peaks	Relative retention time	Theoretical charge at physiological pH
(1)	None	None	$C_{92}O_{46}H_{148}$ (M = 1988.9)	2012 (100%)	$(M+Na)^+$	1.00	-
(2)	Glycine	Glucuronic acid carboxyl	$C_{94}O_{47}N_1H_{151}$ (M = 2045.9)	2085 (100%) 2069 (85%)	$(M+K)^+$ $(M+Na)^+$	0.97	-
(3)	Ethylamine	Glucuronic acid carboxyl	$C_{94}O_{45}N_1H_{153}$ (M = 2016.0)	2039 (100%)	$(M+Na)^+$	1.08	0
(4)	Ethylenediamine	Glucuronic acid carboxyl	$C_{94}O_{45}N_2H_{154}$ (M = 2031.0)	2054 (100%)	$(M+Na)^+$	0.80	+
(5)	Glycine	Aldehyde	$C_{94}O_{47}N_1H_{153}$ (M = 2048.0)	2071 (100%)	$(M+Na)^+$	0.93	-
(6)	Ethylamine	Aldehyde	$C_{94}O_{45}N_1H_{155}$ (M = 2018.0)	2019 (100%)	$(M+H)^+$	0.90	0
(7)	Ethylenediamine	Aldehyde	$C_{94}O_{45}N_2H_{156}$ (M = 2033.0)	2034 (100%)	$(M+H)^+$	0.78	+

[a]Theoretical formula weight was calculated from the exact mass of the commonest isotope of each element. [b]The m/z values for the most predominant peaks noted in fast atom bombardment-mass spectra are reported. The relative intensities of these peaks (expressed as % of the intensity of the most predominant peak) are reported in parentheses. The most predominant peak for QS-21 and most derivatives was the sodium adduct $(M+Na)^+$

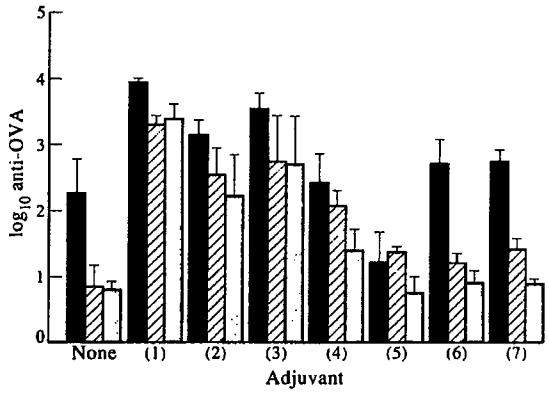

Figure 2 Antigen-specific antibody stimulation by QS-21 derivatives. C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10 and 12 weeks of age with test formulations containing 25 μg OVA adjuvanted with 10 μg QS-21 or QS-21 derivative. A control group immunized with OVA in saline was included. Serum were collected one week after the third immunization and analyzed for anti-OVA of the IgG subclasses IgG1 (solid bars), $IgG2_b$ (cross-hatched bars), and $IgG2_a$ (stippled bars). Data are expressed as the mean and 1 standard error of the log 10 titer of the five mice in each group

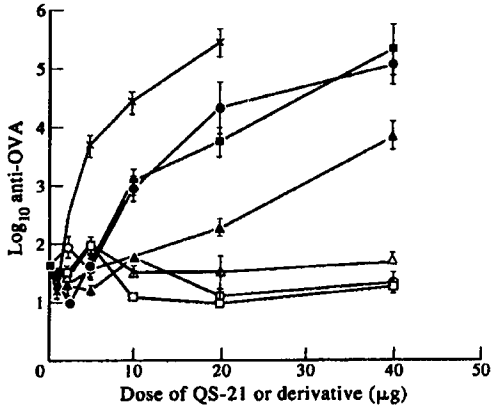

Figure 3 Effect of QS-21 derivative dose on antibody stimulation. C57BL/6 mice (10 per group) were immunized subcutaneously with 25 μg OVA and the indicated dose of QS-21 or QS-21 derivative in a total volume of 0.2 ml saline at 8 and 10 weeks of age. Sera were collected 1 week after the second immunization and an equivolume pool was made from each of the mice in a group; these pools were analyzed in quadruplicate by EIA on plates coated with OVA. Data are reported as the mean and 1 standard error of log_{10} titer. Test adjuvant:(1), X; (2), ●; (3), ■; (4), ▲; (5), ○; (6), □; (7), △

saline formulation, suggesting that these derivatives were inactive as adjuvants. The total IgG titer (not shown) also indicated the same trend.

The anti-OVA total IgG response to different doses of derivatives were compared in *Figure 3*. Mice were immunized subcutaneously with test vaccines containing 25 μg OVA and doses of QS-21 or derivative ranging between 2.5 and 40 μg. Serum was collected at 1 week after a second immunization and tested for total IgG to OVA. QS-21 stimulated anti-OVA IgG at doses between 5 and 10 μg, with some partial effect observed at 2.5 μg. A higher dose was required for QS-21 derivatives which were modified at the glucuronic acid carboxyl ((2)–(4)). However, despite the increase in minimum effective dose, all derivatives modified at the carboxyl retained the function of antibody stimulation. In addition to total IgG, antigen-specific IgG1, $IgG2_b$, and $IgG2_a$ were measured for these derivatives and were observed to increase according to the same dose response curves as for the total IgG (data not shown). In contrast, none of the derivatives prepared by conjugation to the triterpene aldehyde ((5)–(7)) stimulated any increase in antibody titer despite doses of up to 40 μg (approximately 16-fold higher than the lowest dose of QS-21 (2.5 μg) that stimulates any significant increase in titer). Hence, modification of the triterpene aldehyde of QS-21 effectively eliminated its property of antibody stimulation in this dose range, although activity at a higher dose could not be ruled out.

Class I-restricted cytotoxic T-lymphocyte stimulation

One of the more unique properties of QS-21 adjuvant is its activity for stimulation of MHC class I-restricted CTL in response to subunit vaccines. Hence, the derivatives were tested for stimulation of CTL to determine whether modification affects this property. The results are summarized in *Figure 4*. An OVA-transfected syngeneic cell (E.G7-OVA) was used as target. Splenocytes (from mice receiving 3 immunizations with test vaccines) were stimulated by mitomycin-C treated E.G7-OVA to induce CTL maturation; these splenocytes were then used as effector cells in the lysis assay (panel A). The specific killing induced by effector cells from mice receiving derivatives as adjuvants were compared to effectors from mice receiving QS-21 and was used as a measure of precursor CTL induced by these adjuvants. Additionally, denatured OVA was tested as an antigen stimulus to determine whether the derivatives induced an APC population with capacity for antigen processing (panel B).

Derivative (3) (prepared by modification of the glucuronic acid carboxyl with ethylamine) was as effective as QS-21 in stimulating precursor CTL that could be stimulated to mature by stimulation with mitomycin-C treated E.G7-OVA cells. Derivative (3) also induced precursor CTL that could be expanded by denatured OVA, although to a lesser extent than QS-21. Derivatives (2) and (4) (prepared by modification of the glucuronic acid carboxyl with glycine and ethylenediamine, respectively) also stimulated a CTL response. However, this response was lower than that induced by QS-21 and was observed only by use of processed antigen (mitomycin C-treated E.G7-OVA cells) as an antigen stimulus. Doses higher than 10 μg were not tested, so the possibility that these derivatives would induce a stronger lytic response at doses yielding maximum response in antibody stimulation cannot be ruled out. Derivatives (5)–(7) (prepared by modification of the triterpene aldehyde) were not active or poorly active in CTL induction, invoking responses that were similar to that induced by a nonadjuvanted OVA vaccine.

Effect of modification on detergent properties of QS-21

QS-21 and other saponins from *Quillaja saponaria* associate in micelles due to their amphipathic structure. As a measure of the propensity of these molecules to form micelles, the critical micellar concentration was determined (*Table 2*). The critical micellar concentration

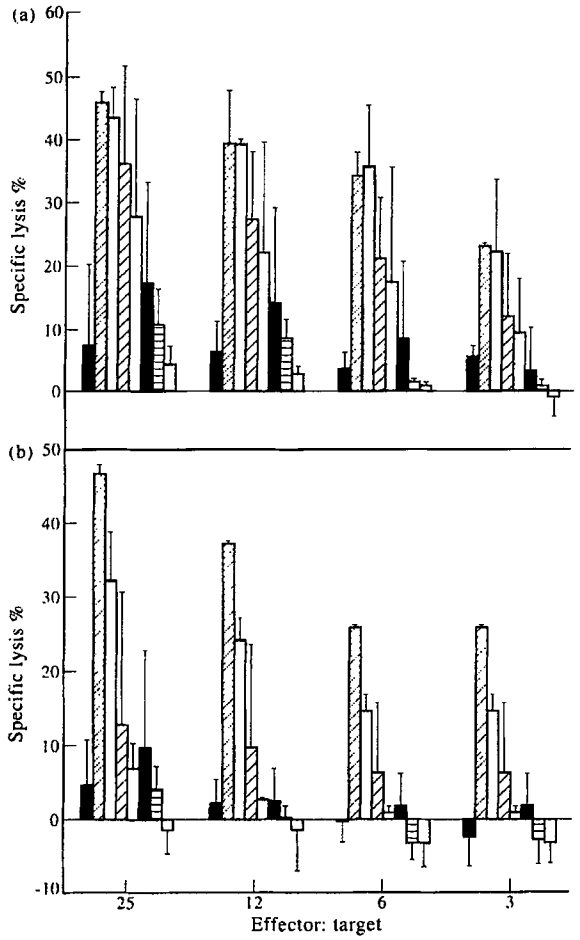

Figure 4 Cytotoxic T-lymphocyte stimulation by QS-21 derivatives. C57BL/6 mice (5 per group) were immunized subcutaneously at 8, 10, and 12 weeks of age with test formulations containing 25 μg OVA adjuvanted with 10 μg QS-21 or QS-21 derivative. CTL activity was measured using E.G7-OVA cells and EL-4 cells as targets and splenic mononuclear cells from immunized animals as CTL effector cells. Splenic mononuclear cells were collected from 2 to 4 weeks after the last immunization. Maturation of precursor CTL to functional effector cells *in vitro* was carried out by specific antigen stimulation using mitomycin C-treated E.G7-OVA cells (panel A) and denatured OVA (panel B). Data are expressed as mean % specific lysis +1 standard deviation from two separate assays of pooled splenocytes from 2 or 3 mice after subtraction of background lysis of EL4 cells. Test adjuvant: None ▨; QS-21, (1), □; (3), ▨; (4), □; (2), □; (6), □; (7), ▤; (5), □

was minimally affected by modification of the glucuronic acid, with these derivatives having lower cmc values than the intact QS-21. This was consistent with the site of modification, which is in one of the hydrophilic domains of the QS-21 molecule. In contrast, the cmc values of the derivatives modified at the aldehyde were substantially higher than that of QS-21, ranging from 2.3-fold higher for the cationic ethylenediamine derivative to almost sixfold higher for the anionic glycine derivative, consistent with modification of QS-21 in one of the two hydrophobic domains expected to contribute to association.

The lysis of red blood cells in an *in vitro* assay was used as a second measure of detergent activity. The relative hemolytic activity of the derivatives was measured and compared to that of unmodified QS-21

Table 2 Detergent properties of QS-21 derivatives

Derivative	Critical micellar concentration (μM)[a]	Concentration inducing 50% hemolysis (μM)[b]
QS-21 (1)	26.6±4.9	4
(2)	22.0±7.7	16
(3)	16.0±2.2	7
(4)	13.3±8.2	17
(5)	147±2.1	>244[c]
(6)	89.6±0.9	>248
(7)	61.0±8.2	>246

[a]The critical micellar concentration was determined in phosphate-buffered saline, pH 7.0, as described in Materials and Methods. Data is expressed as mean ±1 standard deviation in assay of two separate preparations of each derivative. [b]The concentration inducing 50% hemolysis of sheep erythrocytes in an *in vitro* assay was determined as described in Materials and Methods. [c]Highest concentration tested

(*Table 2*). Modifications made at the glucuronic acid did not substantially affect the hemolytic activity. However, modifications at the triterpene aldehyde eliminated hemolytic activity up to the highest concentration tested (244–248 μM). The results were consistent with the results from the critical micellar concentration determination, indicating that the lysis of cellular membranes was affected by modifications that increased the cmc. For QS-21 and those derivatives that retained hemolytic activity, minimum hemolytic concentrations were lower than the cmc, indicating that the monomeric form of QS-21 and derivatives is the form which associates with the erythrocyte membrane.

DISCUSSION

All derivatives were tested for adjuvant activity by determining their effects on both antibody response and cellular mediated response to determine whether these activities were affected equally or would be affected in an unequal fashion. Augmentation of antibody but not CTL or the converse would suggest that different QS-21 functional groups or regions are involved in these separate activities. However, derivatives prepared by modification at the glucuronic acid carboxyl were active for stimulation of both antibody and CTL; derivatives prepared by modification of the triterpene aldehyde were inactive for both responses. This does not rule out the possibility that these activities can be mapped to separate regions that we have not yet examined. For example, diphosphoryllipid A has both adjuvant and toxic properties, but the derivative monophosphoryllipid A retains adjuvant activity, but exhibits significantly lower toxicity than the parent molecule[18].

Some moderate association between detergent activity and adjuvant activity was noted in this study. This was most evident in the three derivatives which were modified at the triterpene aldehyde. This modification resulted in substantial increases in both the critical micellar concentration and in concentrations required for hemolytic activities, indicating that the modification of the apolar triterpene interferes with self-association and membrane association. This was correlated with a loss of adjuvant activity. By contrast, modification of glucuronic acid, which is part of the hydrophilic glycoside and is not expected to participate in self-association,

did not substantially affect the detergent properties of this molecule and adjuvant activity was retained at high doses. However, this correlation between loss of hemolytic activity and loss of adjuvant activity is different from our previous observation that a naturally occurring *Quillaja saponaria* saponin, QS-7, is not hemolytic up to 500 μg ml^{-1}, but is adjuvant active[3]. Derivative (3) (conjugation of ethylamine to the glucuronic acid) is more hydrophobic than QS-21, has a lower critical micellar concentration than QS-21, and has similar hemolytic activity. However, the minimum effective dose of (3) indicated by the dose response for stimulation of antibody was several fold higher than native QS-21. We have previously observed that QS-21 is active as an adjuvant below the critical micellar concentration[17]. Hence, the adjuvant properties of QS-21 are not necessarily associated with its lysis of cell membranes or its properties of self association or membrane association.

Studies with muramyldipeptide (MDP) derivatives such as MTP-PE suggest that increasing hydrophobicity does not substantially affect antibody stimulation, but improves cell-mediated responses[19]. Lipophilicity was also noted to be important for the adjuvant effect of nonionic block copolymer adjuvants[20]. However, lipophilic derivative (3) of QS-21 was not improved over QS-21 in either minimum dose and levels of antibody stimulation or induction of cell-mediated immune responses, measured in this study as induction of Class I restricted CTL. However, it did induce the highest CTL response of the derivatives, suggesting that an increase in lipophilicity does influence this function. Although most of the derivatives were apparently less hydrophobic than QS-21, being retained less tightly on reversed-phase HPLC, the range of retention times of these derivatives was encompassed by that of naturally occurring, adjuvant-active, polar saponins from *Quillaja saponaria* such as QS-7, QS-17 and QS-18, which stimulate antibody response in mice in the same dose range as QS-21[3,4]. Hence, we expected that any differences observed in biological function of these derivatives would be primarily due to blocking of the functional groups, with the polarity changes playing a relatively minor role in the differences.

The three derivatives prepared by conjugation of small molecules to the glucuronic acid carboxyl retained substantial adjuvant activity. In addition to stimulating total antigen-specific IgG titer, these derivatives were active in stimulating antigen-specific IgG1, $IgG2_b$ and $IgG2_a$, suggesting a stimulation of both Th1 and Th2 cells. The ability to stimulate $IgG2_b$ and $IgG2_a$ antibody has been noted previously for unmodified QS-21[3,4], suggesting that modification at the glucuronic acid does not affect the Th1-type response associated with QS-21. Furthermore, these derivatives were active as adjuvants for induction of MHC Class I-restricted precursor CTL. Immunization with antigen/QS-21 has been suggested to set up an activated macrophage population that is highly efficient in the presentation of exogenously provided antigen such as OVA to the Class I MHC pathway[21]. One of the derivatives appeared to retain this property. The effector cells from mice immunized with OVA and derivative (3), modified at the glucuronic acid with ethylamine, were stimulated to produce mature CTL after stimulation with denatured OVA, suggesting that this derivative retained the ability of the native QS-21 molecule for activation of this APC population for processing and presenting OVA antigen. Hence, derivatives modified at the glucuronic acid carboxyl retained the ability to set up the same type of immunological responses as the native QS-21. This was in spite of modifications that blocked the anionic carboxyl group with a neutral or cationic molecule. Hence, it is unlikely that this functional group is directly involved in adjuvant function. However, the increase in minimum effective dose with these derivatives relative to QS-21 suggests a potential steric hindrance of a site important to activity. It has been proposed that the glucuronic acid on *Quillaja* saponins prevents aggregation of immune-stimulating complexes (ISCOM) containing *Quillaja* saponin[22]. Because we were able to utilize the QS-21 derivatives as adjuvants in soluble form, we did not attempt to prepare ISCOM with these QS-21 derivatives to determine whether the elimination of the charge affected ISCOM formation.

The glucuronic acid site is a potential site for conjugation directly to antigen. Conjugation of muramyl dipeptide adjuvant directly to either luteinizing hormone-releasing hormone[23] or to coliphage MS-2 viral peptide coupled to a polymeric carrier[24] yielded highly immunogenic complexes in the absence of additional adjuvant. This strategy could also be used with QS-21, potentially decreasing the required amount of antigen and/or adjuvant in a vaccine formulation. We have already demonstrated that QS-21 can be directly coupled through an amide linkage through the glucuronic acid carboxyl to free amino groups on lysozyme, resulting in a 1:1 molar conjugate that induces higher antibody titers to lysozyme than free lysozyme and QS-21[4]. Additional studies are ongoing to analyze both antibody and CTL responses to a 1:1 molar covalent conjugate of OVA: QS-21.

In contrast to modification of glucuronic acid, the modification of the aldehyde at C4 on the QS-21 triterpene severely diminished adjuvant activity. All three derivatives modified at the aldehyde were inactive as adjuvants over a dose range 10-fold higher than the minimum dose of native QS-21 associated with some stimulatory effect (2.5 μg). Hence, this aldehyde may be critical to the adjuvant function. One possible mechanism involving the aldehyde might be the formation of a Schiff base with a free amino group on a cellular target to stabilize a cellular interaction. Stabilization of interaction of MHC Class II+ antigen-presenting cells and T_h cells via Schiff base interaction between free amino groups on antigen-presenting cells and aldehyde on the T cells has been noted[25]. The inactivation of the QS-21 adjuvant function by blocking the aldehyde suggests that it may also be involved in a Schiff base interaction with a free amino group on the surface of an immune cell target. However, a direct Schiff base-stabilized interaction of QS-21 with a particular immune cell population has not yet been demonstrated. It was shown through *in vivo* and *in vitro* cell depletion and reconstitution studies that macrophages are critical for both induction of precursor CTL to QS-21/subunit antigen vaccines as well as being critical for antigen processing during CTL maturation[21]. Hence, macrophages may be an important site of action for QS-21. However, other immune cell populations such as T cells cannot be ruled

out. Additional studies are planned to further determine the site of action of QS-21. Upon determination of the immune cells that interact with QS-21, the possible interaction of the aldehyde with these cells will be explored.

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SEPARATION AND CHARACTERIZATION OF SAPONINS WITH ADJUVANT ACTIVITY FROM *Quillaja saponaria* MOLINA CORTEX

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Saponins were purified from *Quillaja saponaria* Molina bark by silica and reverse phase chromatography. The resulting purified saponins were tested for adjuvant activity in mice. Several distinct saponins, designated QS-7, QS-17, QS-18, and QS-21, were demonstrated to boost antibody levels by 100-fold or more when used in mouse immunizations with the Ag BSA and beef liver cytochrome b_5. These purified saponins increased titers in all major IgG subclasses. To determine optimal dose in mice for adjuvant response, QS-7 and QS-21 were tested in a dose-response study in intradermal immunization with BSA in mice; for both of these purified saponins, adjuvant response (determined by stimulation of ELISA titers to BSA) neared maximum at doses of 5 μg and was shown to plateau up to the highest dose tested, 80 μg. These purified saponins vary considerably in their toxicity, as assessed by lethality in mice; the main component, QS-18, being the most toxic. Saponins QS-7 and QS-21 showed no or very low toxicity in mice, respectively. None of these saponins stimulated production of reaginic antibodies. The monosaccharide composition of these saponins showed similar but distinct compositions with all four containing fucose, xylose, galactose, and glucuronic acid. Predominant differences were observed in the quantities of rhamnose, arabinose, and glucose. Monomer m.w. (determined by size exclusion HPLC) were determined to range from 1800 to 2200.

Formulation of effective vaccines requires not only the appropriate Ag, but also the appropriate adjuvant to optimize protective humoral and cell-mediated immune responses. The use of the same Ag with different adjuvants has been shown to elicit significantly different responses from the immune system. For example, comparison of immunization of mice with killed schistosomula from *Schistosoma mansoni* with the adjuvants bacillus Calmette-Guérin, pertussis, *Coryne bacterium parvum*, tetanus toxoid, *Escherichia coli* LPS, yeast glucan, aluminum hydroxide, and saponin showed that only the animals immunized with bacillus Calmette-Guérin or saponin were protected from challenge (1) despite the demonstration of significant humoral immunity by some of the ineffective adjuvants. In effect, Allison et al. have noted that adjuvants such as aluminum hydroxide and mineral oil produce primarily humoral immunity whereas adjuvants such as muramyl dipeptide are able to induce cell-mediated immunity as well as differences in the isotype of the antibodies elicited (2). A further consideration, in addition to the efficacy of the adjuvant for eliciting a protective immune response, is the issue of toxicity of the adjuvant. CFA, which is used widely in research vaccines, produces excellent humoral and cell-mediated immunity, but is unsuitable for use in human and veterinary vaccines because of the toxic side effects (3). Similarly LPS, which is also a strong adjuvant, is highly toxic (reviewed in Reference 4). Hence, there is a need for identification of adjuvants that are both safe and efficacious.

One such potential adjuvant system is a class of compounds extracted from plant sources, termed collectively as saponins because of the detergent properties associated with them. The detergent properties of saponins are caused by their amphipathic nature; they consist of a hydrophilic carbohydrate moiety and a hydrophobic steroid or triterpene moiety. The adjuvant effect of saponins was noted in 1951 by Espinet (5) who utilized a crude saponin mixture to increase the immune response to foot-and-mouth disease vaccine. Extracts of the bark of a South American tree, *Quillaja saponaria* Molina, have been shown to be potent adjuvants (6–8). Further studies by Dalsgaard showed that adjuvant activity in these extracts resides in the saponin fraction, which has been characterized as a mixture of triterpene glycosides (7). Crude preparations of *Quillaja* saponins have been used to boost the response to BSA (7), keyhole limpet hemocyanin (9), SRBC (8), as well as aluminum hydroxide-based vaccines (9, 10). In addition, partially purified *Quillaja* saponins have been reported to associate with hydrophobic or amphipathic proteins and lipids to form detergent/lipid/saponin complexes termed ISCOM[4] (11); these structures are typically prepared by solubilizing the Ag with non-ionic detergents and then exchanging the non-ionic for the saponin detergent by centrifugation through sucrose gradients containing saponins at a concentration higher than their critical micellar concentration. ISCOM, which have been prepared from surface Ag isolated from influenza virus, measles, toxoplasma, feline leukemia virus, EBV, and HIV-1 (11–13) induce

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[4] Abbreviations used in this paper: ISCOM, immunostimulating complexes; MDP, muramyl dipeptide; MPL, monophosphoryl lipid A; TDM, trehalose dimycolate; TFA, trifluoroacetic acid; I.D., inside diameter.

serum antibody titers that are approximately 10-fold higher than immunization with protein micelles alone.

In addition to the potent adjuvant activity, the saponin fraction from *Quillaja* bark has strong hemolytic activity (7). This hemolytic activity has been suggested to be caused by the intercalation of saponins into cholesterol-containing membranes to form holes of approximately 80 Å, which can be observed with negative staining electron microscopy (14–16).

Despite the potential use of *Quillaja* saponins as adjuvants, their application has been limited because of the undesirable side effects of the commercially available preparations that are partially purified mixtures of saponins and other components (17–20). The adjuvant-active saponins have not been characterized because of the difficulty in purifying the active components to homogeneity. An adjuvant-active fraction was prepared from an aqueous extract of *Q. saponaria* bark by Dalsgaard (7) by using dialysis, anion exchange, and gel filtration chromatography in aqueous buffers; this fraction (designated Quil-A) was reported to be a single band by TLC on silica gel plates. However, we have found that this fraction is still a heterogeneous saponin mixture that can be resolved into multiple glycoside fractions by reverse phase HPLC. Higuchi et al. (21) have recently substantially purified a saponin from a methanolic extract of *Quillaja* bark and have characterized the glycoside moiety; however, this purified saponin was not tested for adjuvant effect. Hence, at present, there is no information on which components of the saponin fraction from *Quillaja* bark possess adjuvant activity. In this paper, we report a separation procedure for saponins extracted from the cortex of *Q. saponaria* Molina, identification of distinct saponin components with adjuvant activity and no apparent lethality in mice in an adjuvant-active dose range, identification of an adjuvant-saponin that is lethal at a lower dose than the original aqueous extract, and preliminary chemical characterization of these fractions.

MATERIALS AND METHODS

Purification of saponins. Coarsely chopped *Q. saponaria* bark (approximately 1 cm square, obtained from Hauser Chemicals, Boulder, CO) was stirred with 10 ml of water/g of bark at room temperature for 1 h. The extract was centrifuged and the supernatant containing the solubilized saponins was saved. The extraction step was repeated on the bark pellet and the two supernatants were pooled. To remove nonsaponin components, the supernatant pool was lyophilized, redissolved in 40 mM acetic acid in water at a concentration of 250 mg/ml (w/v) and either chromatographed through Sephadex G-50 (medium, Pharmacia, Piscataway, NJ) in 40 mM acetic acid with the hemolytic activity localized in the void volume fraction, or dialyzed against 40 mM acetic acid with the hemolytic activity retained by the dialysis membrane.

The hemolytic fraction was lyophilized and redissolved at a concentration of 200 mg/ml in 40 mM acetic acid in chloroform/methanol/water (62/32/6, v/v/v); 1 g of this fraction was applied to Silica Lichroprep (E. M. Science, Gibbston, NJ; 40 to 63 μm particle size, 2.5 cm I.D. × 20 cm height) and eluted isocratically in the solvent used to solubilize the saponins. The elution of saponins was monitored by carbohydrate assay (22). Fractions containing the saponins of interest were identified by reverse phase TLC with visualization with Bial's reagent (Sigma, St. Louis, MO) pooled individually, and rotavapped to dryness. The fractions from the silica chromatography were then redissolved in 40 mM acetic acid in 50% methanol and loaded on a semipreparative HPLC column (Vydac C_4, 5 μm particle size, 3000 nm pore size, 10 mm I.D. × 25 cm length). Saponin peaks, detected by absorbance at 214 nm, were eluted by using a methanol gradient at a flow rate of 4 ml/min, and individually rotavapped to dryness. Purity of saponins was assessed by analytic HPLC (Vydac C_4, 5 μm particle size, 3000 nm pore size, 4.6 mm I.D. × 25 cm length) with a gradient of 0.1% TFA in acetonitrile.

Immunologic procedures. CD-1 mice (8 to 10 wk of age) were immunized intradermally with a total volume of 0.2 ml injected at two sites per mouse. Each sample was tested in a group of five mice. The buffer used for all immunizations was PBS. The following proteins were used as Ag: BSA (Sigma) and purified cytochrome b_5 from beef liver, kindly provided by Dr. Philipp Strittmatter (University of Connecticut Health Center, Farmington, CT). CFA and IFA were obtained from Difco (Detroit, MI). MPL and TDM were obtained from Ribi Immunochemicals (Hamilton, MT). Squalene and Tween-20 were obtained from Sigma. Superfos Quil-A, a crudely enriched saponin preparation, and Alhydrogel (2% aluminum hydroxide) were obtained from Accurate Sciences, Westbury, NY.

The toxicities of Quil-A and purified saponins QS-7, 18, and 21, were tested in CD-1 mice by following procedures similar to those described above for immunizations. Varying doses of these compounds dissolved in sterile PBS were injected intradermally in mice. The mice were monitored for 72 h after injections and the results expressed in number of deaths per group.

Ag-specific antibody response was determined by ELISA. Immulon II plates were coated overnight at 4°C with 100 μl/well of coating solution, consisting of 10 μg/ml of the Ag in PBS. Plates were then washed twice with PBS and blocked in 10% normal goat serum (Hazelton, Rockville, MD) in PBS (150 μl/well for 1 h at room temperature). Plates were washed twice with 0.05% Tween 20 (Sigma) in water. Mouse serum was serially diluted 1/10 in 10% normal goat serum in PBS; 100 μl of each dilution was incubated on the plate for 1 h at room temperature. All dilutions were tested in duplicate on both Ag-coated and noncoated control wells. Plates were washed twice with 0.05% Tween 20. Goat anti-mouse IgG-horseradish peroxidase conjugate (H and L chain specific; Boehringer-Mannheim Indianapolis, IN), diluted in 10% normal goat serum in PBS, was incubated on the plate (100 μl/well for 30 min at room temperature). The plates were washed four times with 0.05% Tween 20 and then with water two times. The substrate for the reaction was tetramethylbenzidine (23). Titers were determined from the dilution resulting in an absorbance of 0.5. Relative titers of specific antibody isotypes were determined by titration of sera pools (prepared with equivolume ratios of individual mouse serum samples in a group) on Ag-coated plates with the use of goat anti-mouse alkaline phosphatase conjugates specific for IgM, IgG3, IgG1, $IgG2_a$, and $IgG2_b$, respectively (Southern Biotechnology Associates, Birmingham, AL) and a goat anti-mouse IgE-horseradish peroxidase conjugate (Nordic, El Toro, CA).

Hemolytic activity. Serial 1/2 dilutions of saponin in PBS were made in a round bottom microtiter plate. The final volume in each well was 100 μl. SRBC (40% sheep blood and 60% Alsever's solution; Whittaker Bioproducts, Walkersville, MD) were washed three times by low speed centrifugation of the blood followed by resuspension of the red cell pellet in PBS to the original volume. The red cell pellet was diluted to 2.5 × the original volume and then used in the hemolysis assay. Twenty-five microliters of the resuspended cells were added to each well in the microtiter plate and mixed by pipetting. After incubation at room temperature for 30 min, the plates were spun at 1000 rpm for 5 min in a Sorvall RT6000 in an H-1000 rotor to sediment unhemolyzed cells. Fifty microliters of the supernatant from each well were transferred to the same well of a flat bottom microtiter plate. Absorbance caused by released hemoglobin was determined at 570 nm with a Dynatech microtiter plate reader.

Carbohydrate analysis. Relative carbohydrate concentration was determined by the anthrone method of Scott and Melvin (22). The standard for the assay was glucose. Analysis of carbohydrate composition as trimethylglucosides was carried out under contract by the Complex Carbohydrate Corporation (Athens, GA).

Monomer size of saponins. Monomer size of the saponins was determined by HPLC gel permeation chromatography on a Zorbax PSM 60 Si column (6.2 mm I.D. × 25 cm height). Ginsenoside Rb_1 (m.w. = 1109; Waco Pure Chemicals, Dallas, TX) and 18-β-glycyrrhetinic acid (m.w. = 471; Fluka Chemicals, Everett, WA) were used as m.w. standards. Saponins and standards were solubilized in methanol at a concentration of 1 mg/ml. Twenty microliters were injected on the column and eluted in methanol at a flow rate of 1.0 ml/min. Absorbance at 214 nm was used to monitor the column.

RESULTS

Isolation and characterization of saponin adjuvants. Approximately 20 to 25% of the dry weight of *Q. saponaria* Molina bark is extractable in water. Dialysis of the aqueous extract resulted in retention of approximately 24% of the dry weight and 95% of the hemolytic activity of the extract, indicating that saponins present in the

aqueous bark extract were retained by a dialysis membrane of 12,000 m.w. cutoff. Similar recoveries were achieved by chromatography of the aqueous extract on Sephadex G-50, with the saponin fraction localized in the void volume; reverse phase TLC showed that the identical components were isolated (not shown).

With the use of reverse phase HPLC, an unprocessed extract of *Q. saponaria* bark was shown to be a highly complex mixture. Treatment of this aqueous extract by ultrafiltration through a membrane with 10,000 m.w. cutoff removed almost all hydrophilic peaks from the retentate although multiple hydrophobic components were still present (Fig. 1*A*). Analysis of Quil-A, a commercial saponin that is commonly used in adjuvant studies, showed that this product contains all the peaks present in the ultrafiltrated aqueous bark extract shown in Figure 1*A*.

Significant resolution of the saponin peaks in the ultrafiltration retentate was achieved by using a shallow gradient of methanol or acetonitrile on Vydac C_4 as described in *Materials and Methods* (Fig. 1*A*). All major peaks in this retentate fraction were reactive with anthrone, indicating the presence of carbohydrate, and caused foaminess in aqueous solution, indicating that they were saponin in nature. Different bark samples yielded qualitatively a similar pattern of peaks with the same retention times. However, some quantitative differences were observed between different bark samples, apparently as a result of differences between the bark samples because extractions from the same sample of bark yield consistent results. The saponin peaks isolated by HPLC were tested for adjuvant activity by using BSA as the test Ag. Adjuvant-active components were identified in 10 of the peaks tested including the major peaks (7, 17, 18, and 21) (data not shown). These peaks, particularly peak 18, predominate in most samples of bark or commercial *Quillaja* saponins tested.

Figure 1. HPLC (Vydac C4, 4.6 mm × 25 cm, 5 μm particle size, 3000 nm pore size) of an aqueous bark extract treated by ultrafiltration (*A*), saponin QS-7 (*B*), saponin QS-17 (*C*), saponin QS-18 (*D*), and saponin QS-21 (*E*). Gradient was 30 to 40% 0.1% TFA/acetonitrile/30 min, 40%/15 min at a flow rate of 1 ml/min. A total of 100 μg of purified saponin or 200 μg bark extract (dry weight) was used per injection.

The major saponin peaks, purified as described in *Materials and Methods*, were further characterized for adjuvant activity as well as for physical and chemical properties. The purity of these samples is shown in Figure 1. The fractions, designated as saponins QS-7, 17, 18, and 21, with QS denoting the source to be *Q. saponaria*, are significantly pure in comparison with the starting extract, although several minor contaminants are evident in some fractions (Fig. 1 *B* to *E*).

Effect of dose on adjuvant effect in mice. To establish the range of effectiveness for purified saponins, dose response curves were carried out for two of the saponins, QS-7 and QS-21 (Fig. 2). These saponins were chosen because they represented the most hydrophilic (QS-7) and hydrophobic (QS-21) of the four saponins purified in this study. Hydrophobicity was assumed to be related to the retention time on reverse phase HPLC with the use of a hydrophobic resin. CD-1 mice were immunized intradermally twice with BSA plus the indicated dose of saponin at 2-wk intervals. Sera was analyzed for anti-BSA IgG by ELISA 1 wk after the second immunization. Anti-BSA IgG titers were considerably augmented by doses of saponin as low as 5 μg for both QS-7 and QS-21. The immune responses obtained with QS-7 and QS-21 were similar, reaching a plateau at doses between 10 and 80 μg. No significant differences were observed between QS-7 and QS-21.

Adjuvant activity of purified saponins and research adjuvants. The purified *Quillaja* saponins (QS-7, 17, 18 and 21) were compared for effectiveness as adjuvants with various research adjuvants, such as aluminum hydroxide, CFA, and IFA, and a mixture of MPL and TDM. Saponins were used at a dose of 20 μg, an amount that falls in the plateau of maximum adjuvant effect observed with QS-7 and QS-21. Two immunizations with 10 μg of Ag cytochrome b_5 plus QS-7, 17, 18, or 21 in PBS resulted in an increase of approximately 10^3 in Ag-specific IgG ELISA titers when compared to a control group that received Ag alone. The titers observed in the groups receiving purified saponins were similar to those induced by the MPL/TDM mixture and CFA and IFA. However, purified saponins induced a higher response than aluminum hydroxide (Fig. 3).

Isotype of antibodies augmented by saponins. Adjuvants that augment similar IgG titers may differ considerably in boosting various IgG subclasses. Therefore, the

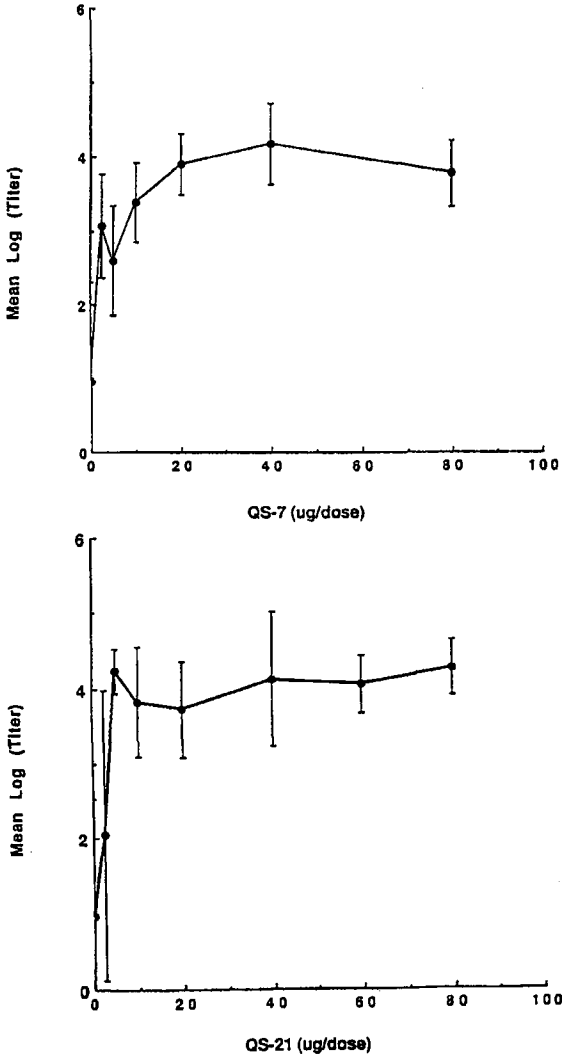

Figure 2. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 5 μg BSA and the indicated dose of QS-7 and QS-21. Results are expressed as means ± SD.

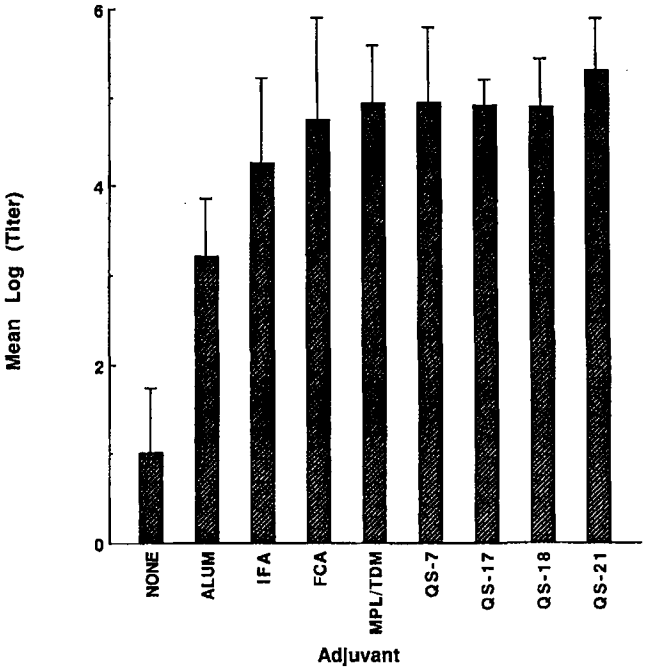

Figure 3. Ag-specific IgG ELISA titers induced in CD-1 mice by two intradermal immunizations with 10 μg cytochrome b_5 and the indicated adjuvant. Formulations adjuvanted with CFA and IFA were prepared by emulsification of 100 μl of Difco CFA or IFA with 100 μl of a PBS/Ag solution/dose. MPL/TDM formulations were prepared by homogenization of 50 μg MPL, 50 μg TDM, 2 μl Squalene, and 0.2 ml 0.2% Tween 20/PBS/Ag/dose. The alum preparation contained 400 μg aluminum hydroxide per dose. The saponin preparations, which were fully soluble in aqueous solution, contained 20 μg of the indicated saponin in 0.2 ml PBS/Ag per dose. Results are expressed as means ± SD.

IgG subclass distribution of the IgG for the immunization experiment described in Figure 3 was determined. After two intradermal immunizations with cytochrome b_5 and saponins QS-7, 17, 18, and 21, antibodies were found in the three major IgG subclasses G1, $G2_b$, and $G2_a$ (Table I). With saponin fractions QS-17, 18 and 21, IgG2a antibodies predominated. In contrast, antibodies induced by Ag in PBS or on aluminum hydroxide were predominantly IgG1. CFA and MPL/TDM adjuvant augmented the production of isotypes IgG1, IgG2a, and IgG2b whereas IFA induced isotypes IgG1 and some IgG2b. In contrast to previous reports with the use of crude saponin preparations from *Q. saponaria* (2), no IgE antibodies were elicited by any of the purified saponins described here. Reaginic antibodies were not detectable at a 1/10 dilution for any of the adjuvants tested. The dose dependence of isotype distribution was not determined.

Purified saponins yielded consistent results in adjuvant effect. Five preparations of QS-21 that had been purified from different sources of *Q. saponaria* Molina bark were tested concurrently in an immunization study with BSA in mice; the mean and SD of the $\log_{10}$ ELISA titer of the five groups receiving three injections of 15 μg of QS-21 and 5 μg of BSA was 4.7 ± 0.13 in comparison with the control group which had a titer of 3.6.

TABLE I

Adjuvant effect on Ag-specific IgG Subclass

Adjuvant	Subclass Titer/Total IgG Titer[a]		
	G1	$G2_b$	$G2_a$
None	1.00	0	0
QS-7	0.35	0.21	0.44
QS-17	0.07	0.21	0.72
QS-18	0.10	0.06	0.84
QS-21	0.15	0.24	0.61
CFA	0.33	0.39	0.27
IFA	0.92	0.07	0.01
Aluminum hydroxide	0.91	0.09	0
MPL + TDM	0.24	0.38	0.38

[a] Sera were obtained at day 35 from the cytochrome b_5 immunization study described in Figure 3.

TABLE II

Lethality of saponins to CD-1 mice[a]

Dose (μg)	Quil-A	QS-7	QS-18	QS-21
125	1/5	0/5	4/5	0/5
250	2/5	0/5	5/5	0/5
500	4/5	0/5	5/5	1/5

[a] Results are expressed as number of deaths per group of five mice within 72 h after intradermal injection of saponins.

Toxic and hemolytic activities. Toxicity (assessed by lethality) has been associated with the use of saponins as adjuvants (20). In effect, the commercial saponin preparation Quil-A was lethal to mice in the dose range of 100 to 125 μg (Table II), as determined with one preparation. The purified saponins described here exhibit a wide range of lethalities. QS-18, the predominant saponin species in

the bark from *Q. saponaria* as well as in commercial preparations such as Quil-A, is the most lethal of those tested with deaths observed at doses as low as 25 μg (data not shown). In contrast, QS-7 is apparently nonlethal up to 500 μg and QS-21 is lethal only at 500 μg, with one mouse dead out of five mice receiving this dose (Table II). In mice, the minimum lethal dose/adjuvant-effective dose ratio is 50-fold for QS-21 and even higher for QS-7. However, the QS-18 adjuvant-effective dose is close to the lethal dose when assayed in mice. Apparently, the lethal effects of Quil-A can be explained in part by the large fraction of QS-18, which is the predominant component in its composition. The variability of QS-18 content in the bark used to prepare Quil-A and other commercial preparations will explain the differences in lethality observed with different preparations. From these results, we can state that there is no relationship between relative adjuvant activity and relative lethality.

The hemolytic activities of the purified adjuvant-saponins were compared. Saponins QS-17, 18, and 21 caused hemolysis of SRBC at concentrations as low as 5 to 30 μg/ml, with concentrations resulting in 50% hemolysis being 25 ± 0 μg/ml, 15 ± 3 μg/ml, and 7 ± 2 μg/ml, respectively (mean and SD of purified preparations derived from two separate bark samples). However, no hemolysis was observed with QS-7 at concentrations up to 200 μg/ml (highest concentration tested). There is no correlation between hemolytic activity, lethality and adjuvant activity, i.e., QS-7, 18 and 21, have approximately the same adjuvant activity but are widely different in hemolytic activity and lethality.

Carbohydrate composition. Purification of saponins allowed a preliminary structural characterization. The analysis of the composition of the four saponins QS-7, 17, 18, and 21 demonstrated the presence of a highly complex glycoside component, consisting of seven or more monosaccharides in saponin QS-7 and eight or nine monosaccharides in saponin QS-17 (Table III). All four saponins contained components with the same linkage, including terminal rhamnose, xylose, galactose, and glucose residues as well as 3-xylose, 2,3-glucuronic acid, and 3,4-rhamnose (linkage data not shown). It appears that these saponins share a common glycoside structure although there are clear deviations in the carbohydrate composition and linkage of the saponins analyzed.

All saponins contain arabinose except for saponin QS-7. Saponin QS-21 contains a diminished amount of glucose, suggesting that this may be caused by a contaminant as it is present in a ratio significantly lower than 1:1 when normalized to galactose. Monomer size of the predominant saponins was estimated by size exclusion HPLC. For comparison, we used triterpene and triterpene glycoside standards of known m.w. This analysis was carried out in methanol to prevent micellization. The monomer size ranges from 1800 to 2200 and is consistent with the m.w. predicted for a triterpene with 8 to 10 monosaccharide residues. It is likely that monosaccharides galactose, glucose, and glucuronic acid are each present in a ratio of 1.0 mol of monosaccharide/mol of saponin as higher molar ratios would significantly increase the m.w.

TABLE III

Molar ratio of monosaccharide/saponin[a]

Monosaccharide	Saponin			
	QS-7	QS-17	QS-18	QS-21
Rhamnose	2.22	2.34	1.15	1.27
Fucose	0.90	0.96	0.88	0.91
Arabinose	Trace	0.98	0.74	0.77
Xylose	1.28	1.33	1.34	1.44
Galactose	1.00	1.00	1.00	1.00
Glucose	1.35	1.23	1.16	0.35
Glucuronic acid	0.65	0.64	0.72	0.74

[a] Determined as trimethylsilated methyl glycosides and normalized to galactose (assumed to be present at 1 mol/mol of saponin).

DISCUSSION

These results demonstrate that the saponin fraction obtained by aqueous extraction of *Q. saponaria* bark is actually a heterogeneous group of related glycosides. All previous attempts to purify adjuvant-active *Quillaja* saponins have been in aqueous solution by methods typically used to purify proteins, such as dialysis, ion exchange chromatography, and size exclusion chromatography (7). Although these methods are useful in partially separating saponins from nonsaponin components, they have been ineffective in separating individual saponins because of the tendency of saponins to form mixed micelles. Hence, effective separation requires the use of organic solvents or solvent/water systems that solubilize the amphiphilic saponins as monomers so that the formation of mixed micelles does not interfere with separation. In effect, adsorption and reverse phase chromatography in organic solvents as described in *Materials and Methods* has allowed the purification of individual saponins to a degree of homogeneity that is significantly higher than that achieved by earlier reports (7, 24).

Although previous reports suggested that exposure to organic solvents destroyed adjuvant activity (25), we were able to recover adjuvant activity by using organic solvents for silica and reverse phase chromatography. The carbohydrate analysis of the individual saponins described in this paper indicate that they consist predominantly of one component, although some heterogeneity is still present because multiple linkage forms of individual monosaccharides can be detected. The carbohydrate composition and linkage analysis of the purified *Quillaja* saponins are similar to that determined by Higuchi et al. for the hydrolytic breakdown products isolated from a partially purified *Quillaja* saponin preparation (26). Dalsgaard reported that the saponin fraction isolated by anion exchange and gel filtration (Quil-A) contained the monosaccharides xylose, arabinose, glucose, rhamnose, and fructose (25) in unspecified ratios. None of the saponins described in this study contain fructose. In addition, they contain monosaccharide residues not reported by Dalsgaard (fucose, galactose, and glucuronic acid).

Adjuvant activity was demonstrated to be associated with several of the saponins, including those that appear to be most predominant, QS-7, 17, 18, and 21. Hence, the adjuvant activity of *Quillaja* bark extracts is associated with several distinct saponins rather than a single component, although the carbohydrate analysis indicates that these saponins may be structurally related. Not all peaks contained components that could serve as adjuvants in our test system.

Saponins QS-7, 17, 18, and 21 were tested more extensively because they were the predominant peaks in most bark samples analyzed. These fractions typically induced an increase in Ag-specific IgG titers when used at doses

ranging from 10 to 20 μg in intradermal immunization in mice. The adjuvant effect of these saponins was observed with both BSA and cytochrome b_5. Evidence that close proximity of Ag and saponin are important for the response was shown by our observation that saponin and BSA injected separately into different flanks of the mice did not induce a boost of Ag-specific IgG titers (data not shown), indicating no apparent systemic response. A similar result has been observed by Bomford (8). The strong antibody response elicited by ISCOM, which are reported to be a complex of saponin, Ag, and lipid (11, 12), are consistent with a close association of Ag and saponin being necessary for the adjuvant response. However, the adjuvant effects of saponins cannot be attributed simply to their detergent properties, i.e., saponin QS-7, which is a poor detergent as revealed by its nonhemolytic properties, has adjuvant characteristics similar to QS-17, 18, or 21, which are highly hemolytic.

Purified saponin adjuvants stimulate an equivalent or higher secondary immune response than that obtained by using aluminum hydroxide, CFA and IFA, or MPL/TDM adjuvants. ELISA titers measured via the end point dilution method, as was done in this study, are thought to be proportional to the concentration of high and medium avidity antibodies (27). Therefore, if it is assumed that the ELISA titers reported here reflect the concentration of these populations, then the purified saponins induce quantities of medium and high avidity IgG comparable with CFA, IFA, and MPL/TDM, and higher than those induced by aluminum hydroxide. However, differences in the concentrations of low avidity antibodies cannot be ruled out. Saponins also influence the Ag-specific isotype profile. A comparison of isotypes produced by mice in response to immunization with purified saponin showed induction of the three major IgG subclasses, G1, $G2_b$, and $G2_a$. The isotype profile observed with these purified saponins differs from that reported by Allison and Byars with a crude saponin (2) in which they found predominantly an IgG1 response to immunization of mice with Ag and crude saponin mixture, a response similar to that elicited by aluminum hydroxide. Under the immunization conditions utilized in this study, saponins induced significant levels of IgG2a and IgG2b as well as G1 antibodies; for some saponins, IgG2a predominated. Ag-specific IgE was not detected, even with the highly toxic QS-18, indicating that other components in crude preparations are responsible for the production of reaginic antibodies.

The high level of protection observed with the use of saponins with vaccines in mice (1) may in part be caused by the ability of saponins to induce an isotype profile similar to that observed in natural immunity arising from a viral or bacterial infection. Viral infections in mice induce an IgG response in which IgG2a accounts for 65 to 92% of total specific antibody (28). IgG2a has also been shown to be protective against protozoal infections (29). Both C fixation and antibody-dependent cellular cytotoxicity in mice can be mediated by IgG2a antibodies (30).

Commercially available saponin preparations are highly heterogeneous mixtures of adjuvant-active and inactive components. The relative concentrations of these components will vary according to the source of the bark, leading to difficulty in preparation of batches with a consistent composition. Substantial variation has been noted between different sources of commercially available saponins (31, 32). Purified saponins can be readily standardized, and this property allows preparation of vaccines with known proportions of a given active saponin or saponins.

The use of purified saponins for immunization allows selection of saponins with the optimal combination of adjuvant activity and negligible lethality. Preliminary studies indicate that some adjuvant-active saponins are significantly more lethal than others when tested at doses over the range of 25 to 500 μg in mice. It may be possible to select an adjuvant-active saponin for use in a vaccine that provides a wider safety margin between adjuvant-active and lethal doses than that in crude saponin extracts (which contains a larger fraction of lethal saponin adjuvants such as QS-18).

No attempt was made to correlate saponin structure with the biologic effects, adjuvant activity, and lethality associated with *Quillaja* saponins. A complete structural determination will involve sequencing of the glycoside moieties, identification of the triterpene, and identification of the point of linkage of the glycoside moieties onto the triterpene backbone. Comparison of the complete structures of naturally occurring variants such as those described here will provide information on what parts of the structure are involved in specific biologic activities. Further information on the minimal structure involved in these activities can be gained by analysis of less complex saponins produced by specific chemical or enzymatic hydrolysis of saponins of known structure. These studies are ongoing.

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EXHIBIT C
(7 pages)

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QS-21 and QS-7: Purified Saponin Adjuvants

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Key words: QS-21, QS-7, adjuvant, *Quillaja saponaria*, saponin, vaccine.

Abstract: QS-21 and QS-7 are two adjuvant-active saponins that can be obtained in high purity from *Quillaja saponaria* Molina extracts. QS-21 is a highly characterized compound and is known to be a potent adjuvant for antibody and $CD8^+$ CTL response to subunit antigens. Less is known about the activity and structure of the hydrophilic saponin QS-7. Hence, we have carried out a detailed structural and immunological characterization. As with QS-21, QS-7 was shown to be a 3,28-O-bisglycoside quillaic acid, with some differences being a higher degree of glycosylation and a considerably shorter fatty acyl unit in QS-7. These differences were correlated to a lower lytic activity against sheep red blood cells. Different doses of QS-7 were evaluated for stimulation of immune response to the antigen ovalbumin, given three times by subcutaneous route to C57BL/6 mice. QS-7 doses of 40 µg or higher were shown to induce a strong $CD8^+$ CTL response reproducibly against E.G7-OVA targets (similar to that induced by a 5 -10 µg dose of QS-21). QS-7 (at doses above 5 µg) was also shown to stimulate CTL against peptide 18 of HIV-1_{IIIB} gp120 after three immunizations of Balb/c mice with recombinant gp120 and different doses of QS-7. These data suggest that a hydrophilic saponin with low lytic activity can stimulate MHC Class I CTL responses although a higher minimum dose may be required for some antigens.

INTRODUCTION

Aqueous extracts of the bark of the South American tree *Quillaja saponaria* Molina contain a potent immunological activity. These extracts are complex mixtures of tannins, polyphenols, and saponins. The adjuvant activity was shown to be associated with the saponin fraction [1]. This fraction consists of many diverse acylated bisdesmodic quillaic acid glycosides. Several predominant *Q. saponaria* saponins were HPLC-purified and identified as adjuvants [2]. One of these purified saponins, QS-21, has been characterized extensively as an adjuvant because of its potent adjuvant activity and low toxicity [3-5]. It is known to stimulate antibody and cytotoxic T lymphocyte responses to subunit vaccines in mice. QS-21 is currently under evaluation in clinical trials with various vaccine antigens [6, 7].

Other saponins from *Q. saponaria* are known to have adjuvant activity for the stimulation of antibody responses. A saponin known as QS-7 is of particular interest as a potential adjuvant. This saponin is more hydrophilic and less lytic to red

blood cells than the other major saponins. It was also shown to have low toxicity, with doses of 0.5 mg being tolerated well by mice. QS-7 was shown to be an adjuvant for antibody responses in mice to the antigens bovine serum albumin and beef liver cytochrome b_5 [2]. However, its potential as an adjuvant for cell mediated immune responses was not explored in previous studies. In this study, we have evaluated the adjuvant effect of QS-7 for induction of cytotoxic T lymphocytes to two antigens in mice, ovalbumin (OVA) and recombinant HIV-1 gp120.

Materials and Methods

QS-7 and QS-21 were purified from an aqueous extract of *Q. saponaria* bark by reversed-phase HPLC. QS-21 and QS-7 were shown to be ≥ 98% and > 95% pure, respectively, when analyzed by reversed phase HPLC analysis on Vydac C4 [5]. The deacylsaponins were prepared by alkaline hydrolysis of aqueous solutions of QS-7 or QS-21, followed by HPLC to isolate the deacylated saponin. Fast atom bombardment mass spectroscopy of purified saponins was carried out at MScan Corp., Westchester, PA. Carbohydrate analysis was carried out by Complex Carbohydrate Corp., Athens, GA.

Immunization was carried out with the antigen OVA in female C57BL/6 mice or with the antigen HIV-1_{IIIB} gp120 in female Balb/c mice. OVA (grade VI) was obtained from Sigma (St. Louis, Missouri). Purified recombinant HIV-1_{IIIB} gp120, expressed by a baculovirus vector in insect cells, was produced as described previously [8]. All mice were 8-10 weeks of age at the start of the first immunization. The antigen doses were 25 μg. Adjuvant doses were varied between 0 to 80 μg. All vaccines were administered by the subcutaneous route at days 0, 14, and 28 of the protocol. Immunological analysis was carried out on splenocytes obtained between days 42 and 56. Cytotoxic T lymphocyte responses were determined as described previously [3]. Haemolytic activity was determined by lysis of sheep red blood cells (SRBC. BioWhittaker, Inc., Walkersville, MD) as described previously [2].

Results

Structural analysis

QS-7 was analysed by fast atom bombardment mass spectroscopy and carbohydrate linkage analysis (Table 1). QS-7 has a smaller molecular weight than QS-21, but is more heavily glycosylated. The main differences in the carbohydrate composition of QS-7 compared to QS-21 were the presence of a terminal glucose, linkage at the 3-position of rhamnose, linkage at the 3-position of fucose, an additional terminal rhamnose, and the absence of arabinose. Mild alkaline hydrolysis is known to convert esterified saponins into deacylsaponins [9]. Hence, we carried out alkaline hydrolysis of the purified saponins and isolated the deacylsaponins to compare the fatty acyl chain of QS-7 with that of QS-21. The molecular weight of the deacylsaponin of QS-21 is 476 lower and does not have arabinose, consistent with hydrolysis of the fatty acid ester bond at the 4-hydroxyl of fucose (Fig. 1). In contrast, mild alkaline hydrolysis of QS-7 to the deacylsaponin does not remove any monosaccharide residues and reduces the molecular weight only by 41. This suggests that QS-7 may be acylated with an acetic acid residue. Both QS-7 and QS-21 were further hydrolysed to the identical prosapogenin (data not shown). A structure of QS-7 that is consistent with the data is shown in Figure 1 and is compared to the known structure of QS-21 [10].

Table 1: Comparison of QS-7 and QS-21.

Saponin	m/z [M+Na]$^+$		Monosaccharide linkage analysis	
	Intact saponin	Deacyl-saponin	Intact saponin	Deacylsaponin
QS-7	1886	1845	t-apiose, t-rhamnose, 3,4-rhamnose, 2,3-fucose, t-xylose, 3-xylose, t-galactose, t-glucose, 2,3-glucuronic acid	Identical to intact saponin
QS-21	2012	1536	t-apiose, 4-rhamnose, 2-fucose, t-xylose, 3-xylose, t-galactose, 2,3-glucuronic acid, t-arabinose	Identical to intact saponin except for loss of arabinose

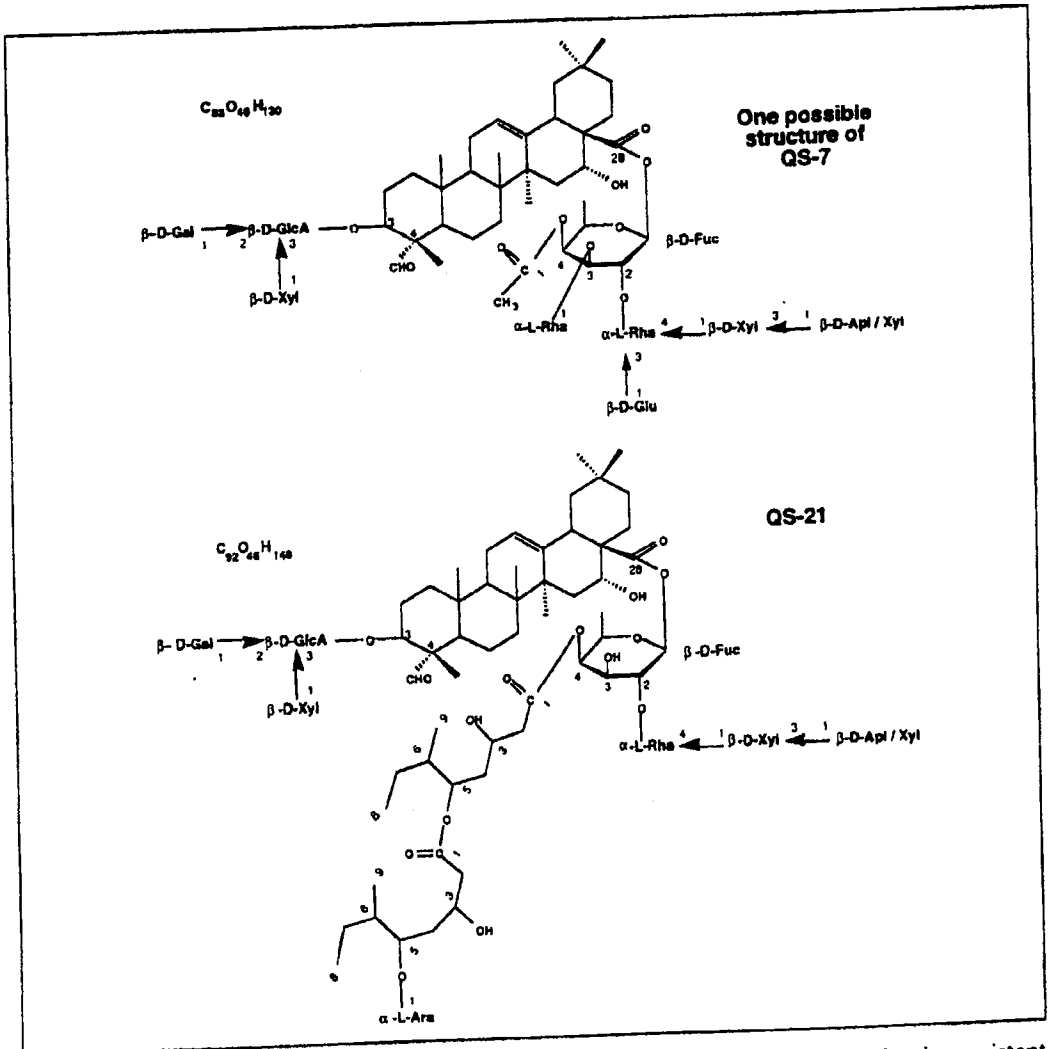

Fig. 1: **Saponin structure.** The structure shown for QS-7 is one possible structure that is consistent with the FAB-MS and carbohydrate analysis. Structure of QS-21 was determined in Jacobsen et al [10].

Adjuvant activity

QS-7 was evaluated as an adjuvant for inducing cytotoxic T lymphocytes to subunit antigens. The antigen ovalbumin (OVA) was chosen as one model because QS-21 is known to induce high CTL responses to OVA [3]. E.G7-OVA cells, which are EL4 mouse T lymphoma cells transfected with the OVA gene [11], were used as target cells. Figure 2 shows that splenocytes from C57BL/6 mice immunized three times with OVA and QS-7 have a strong antigen-specific cytolytic activity. However, a comparatively high dose of QS-7 (40 μg and above) was required to stimulate a CTL response similar to that produced by 5 μg QS-21.

The capacity of QS-7 to stimulate the induction of cytotoxic T lymphocytes was also studied in vaccines consisting of a recombinant glycoprotein, HIV-1_{IIIB} gp120 (Fig. 3). Balb/c mice were immunized subcutaneously three times with gp120 and various doses of QS-7 or QS-21. The CTL response to HIV-1_{IIIB} gp120 was measured as a splenocyte-mediated lysis of P815 cells coated with P18 peptide, the predominant CTL epitope in gp120. In contrast to the high doses of QS-7 required to induce CTL with OVA, lower doses of QS-7 stimulated significant CTL to gp120. QS-7 doses of 5 μg or higher induced CTL responses above background. However, the dose dependence of the response was not clear because the 20 μg dose of QS-7 induced lower CTL response than the 10 μg dose of QS-7. This suggests that these doses are below the minimum dose required for optimal priming for p18-specific CTL.

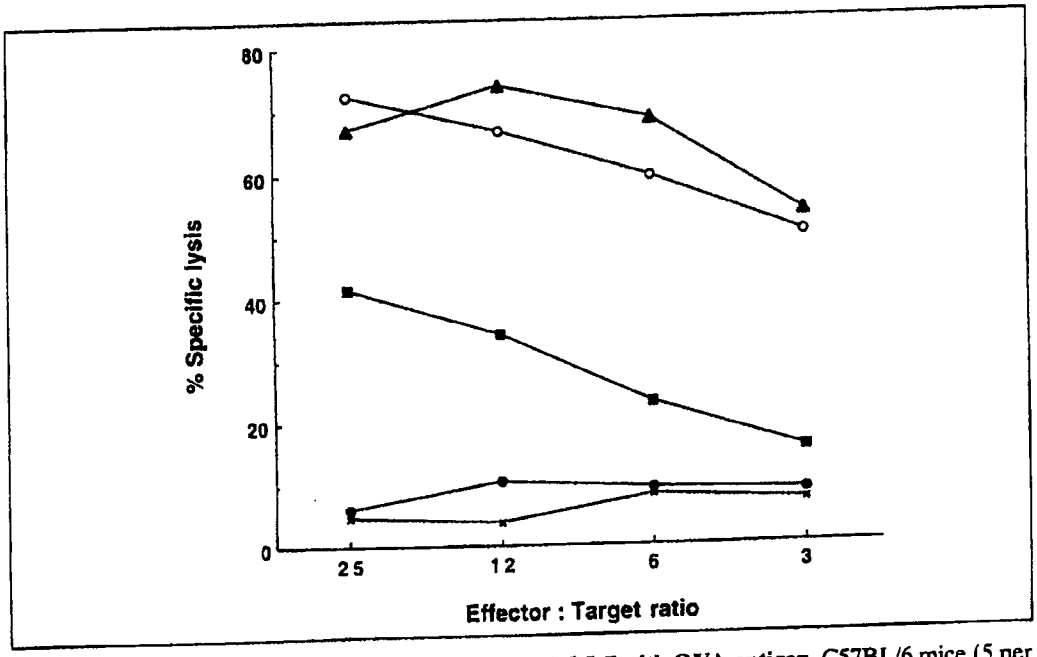

Fig. 2: **Cytotoxic T lymphocyte activity of QS-21 and QS-7 with OVA antigen.** C57BL/6 mice (5 per group) were immunized subcutaneously at days 0, 14, and 28 with 25 μg OVA and different doses of QS-7 or QS-21: no adjuvant (x), 5 μg QS-7 (●), 40 μg QS-7 (■), 60 μg QS-7 (▲), and 5 μg QS-21 (O). Splenocytes were harvested at day 42, stimulated in vitro for six days with mitomycin C-treated E.G7-OVA cells, and used as effector cells in the CTL assay. Lysis was determined by a standard ^{51}Cr release assay on E.G7-OVA cells (EL4 cells transfected with OVA gene and which express $OVA_{257-264}$ on class I MHC). The background lysis of EL4 cells was subtracted.

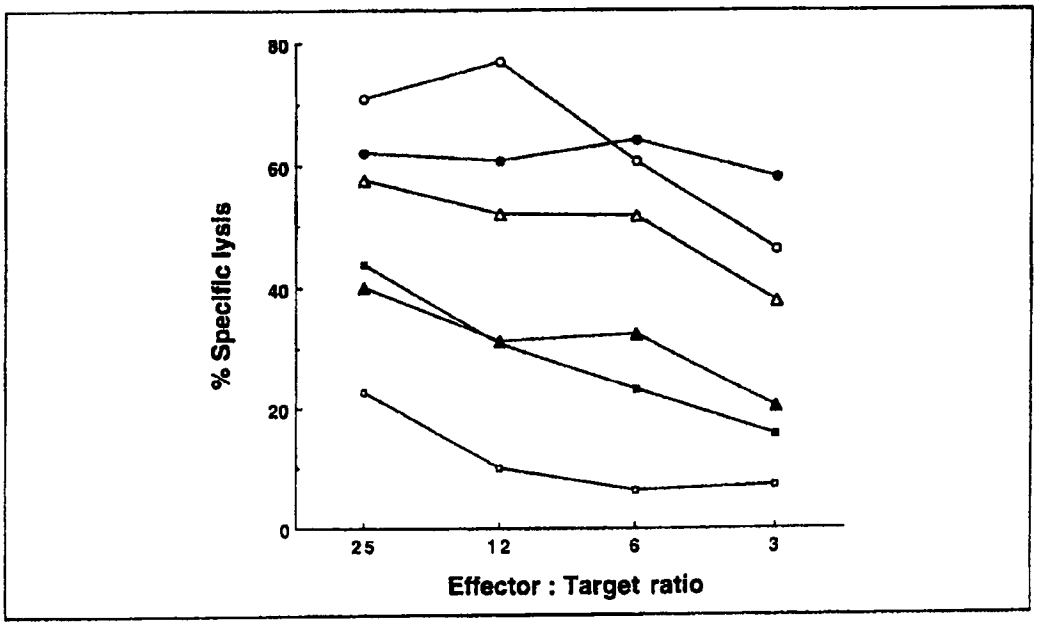

Fig. 3: **Cytotoxic T lymphocyte activity of QS-21 and QS-7 with HIV-1 gp120.** Balb/c mice (5 per group) were immunized subcutaneously at days 0, 14, and 28 with 25 µg gp120 and different doses of QS-7 or QS-21: no adjuvant (—), 5 µg QS-7 (■), 10 µg QS-7 (. ..), 20 µg QS-7 (▲), 40 µg QS-7 (●), and 10 µg QS-21 (O). Splenocytes were harvested at day 42, stimulated in vitro for six days with HIV-1 gp120 peptide 18 (predominant CTL epitope), and used as effector cells in the CTL assay. Lysis was determined by a standard ^{51}Cr release assay on P815 cells coated with the peptide. The background lysis of P815 cells was subtracted.

Lytic activity

One of the properties of most esterified bisdesmodic triterpene saponins is a haemolytic activity. QS-21, at concentrations of approximately 7 µg/ml (3.5 µM), is haemolytic to sheep red blood cells in an in vitro assay [2]. Haemolytic activity is reported to be especially high with esterified saponins. Although QS-7 is an esterified saponin, it has a considerably shorter acyl chain than QS-21. The concentration of QS-7 required for 50% haemolysis was 237 µM.

Discussion

Although many adjuvants are effective in increasing humoral immune responses, fewer adjuvants will improve cell-mediated immune responses, particularly to soluble antigens. Soluble, nonparticulate antigens are not typically processed by the class I major histocompatibility (MHC) antigen pathway that leads to $CD8^+$ CTL responses to antigens synthesized in the cytoplasm («endogenous» antigens), but are instead «exogenous» antigens which are internalized by endocytosis and processed by the class II MHC pathway that leads to antibody responses. Hence, CTL responses are difficult to raise against soluble antigens. However, certain adjuvant and antigen delivery strategies have been shown to modify the

response for soluble antigens from a Class II MHC response to a Class I MHC response. These include antigen delivered in acid-sensitive liposomes [12], lipopeptides [13], and ISCOMs [14]. These may act as lipophilic carriers for directing antigen through the endosomal membrane into the cytosol.

QS-21 is another adjuvant that is known to induce strong $CD8^+$ CTL response to subunit antigens. Although it does not form a complex with soluble, hydrophilic antigens such as OVA, it does interact with cell membranes due to its amphipathic structure. It is one of the more hydrophobic saponins in *Q. saponaria* extracts and is known to be taken up by splenocytes and lymph node cells and will bind to liposomes of phosphatidylcholine/cholesterol (unpublished data). Hence, it is possible that it intercalates into the membranes of APC and enables escape of exogenous antigen into the cytoplasm. Deacylated QS-21 was previously shown to be inactive as an adjuvant for CTL responses [15]. This suggests that esterification may be important for this activity. However, the length of the fatty acid chain may be less important. QS-7, which appears to be esterified with an acetic acid, can clearly stimulate CTL responses. For example, it is an excellent inducer of CTL responses to HIV gp120. With OVA, a several fold higher dose of QS-7 is required to induce a CTL response comparable to that of QS-21. The trend with OVA is that the saponin with higher lytic activity (QS-21) is a stronger inducer of CTL. However, the differences in optimum dose required for induction of CTL to OVA is considerably lower than predicted by the substantial differences in the haemolytic activity between QS-21 and QS-7 (70 fold difference in haemolytic titre). It is possible that there is a stronger correlation between CTL activity and effect of these saponins on membranes of APC than there is with haemolytic activity.

Conclusion

QS-7, a hydrophilic saponin from *Quillaja saponaria*, can stimulate a cell-mediated immune response to HIV-1 gp120 and OVA. Preliminary structural studies suggest that this saponin is a highly glycosylated bisdesmoside quillaic acid saponin, acylated with an acetic acid.

Acknowledgements

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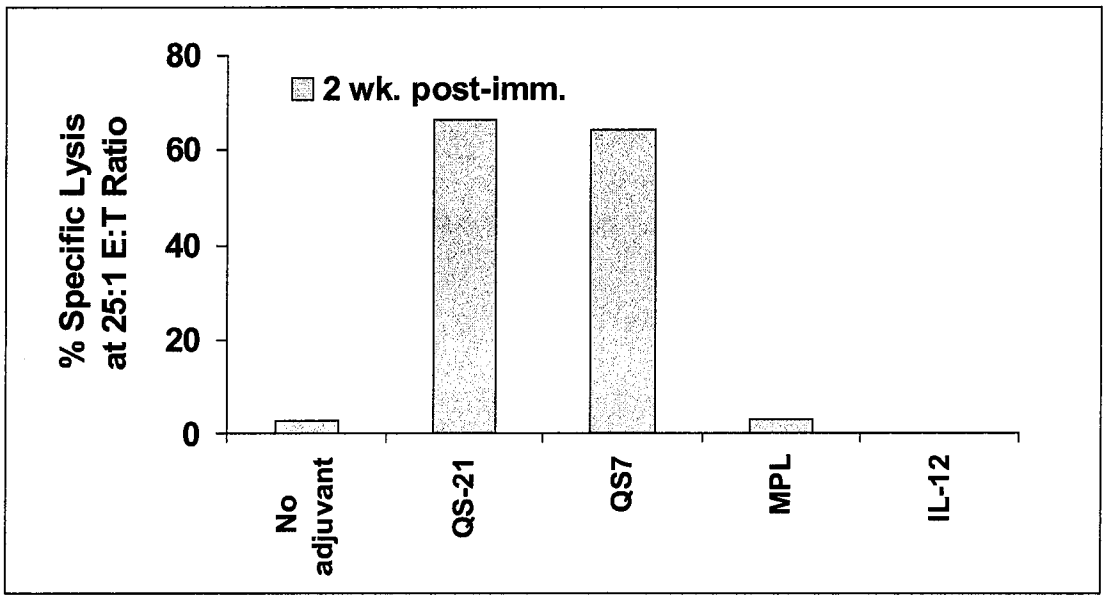
EXHIBIT D
Page 1 of 8

SLIDE A
CTL: QS-21 and QS-7 compared to MPL, IL-12

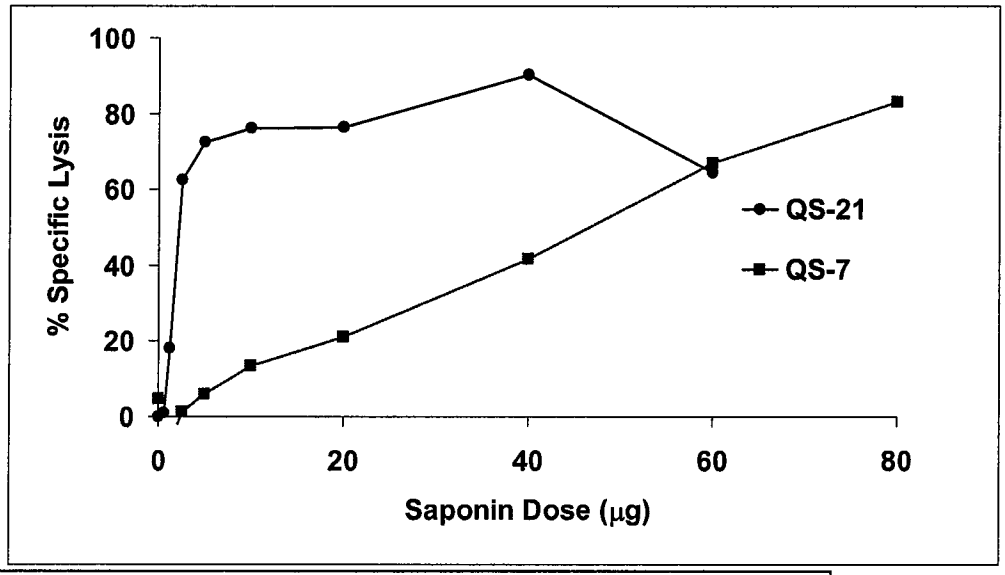


Vaccine = OVA + adjuvant, s.c., days 0, 14, 28
Doses = 10 ug QS-21, 40 ug QS-7, 20 ug MPL, 0.1 ug IL-12
Target cells = E.G7-OVA cells

ANTIGENICS

EXHIBIT D
Page 2 of 8

SLIDE B
QS-21 and QS-7 Dose Response for CTL to OVA

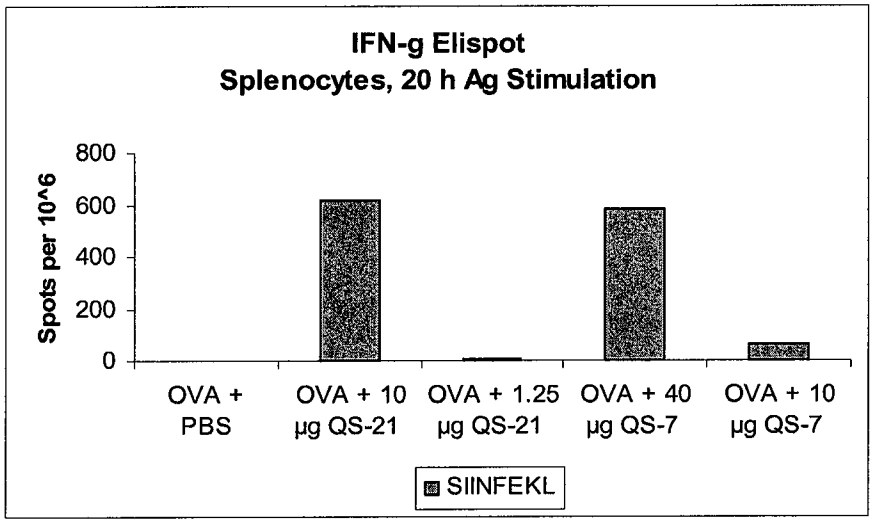


Vaccine = Ovalbumin
3 subcutaneous immunizations in C57BL/6 mice
Effector cells: OVA-stimulated splenocytes
Target cells = E.G7-OVA cells.
Effector: Target Ratio = 25:1

ANTIGENICS

EXHIBIT D
Page 3 of 8

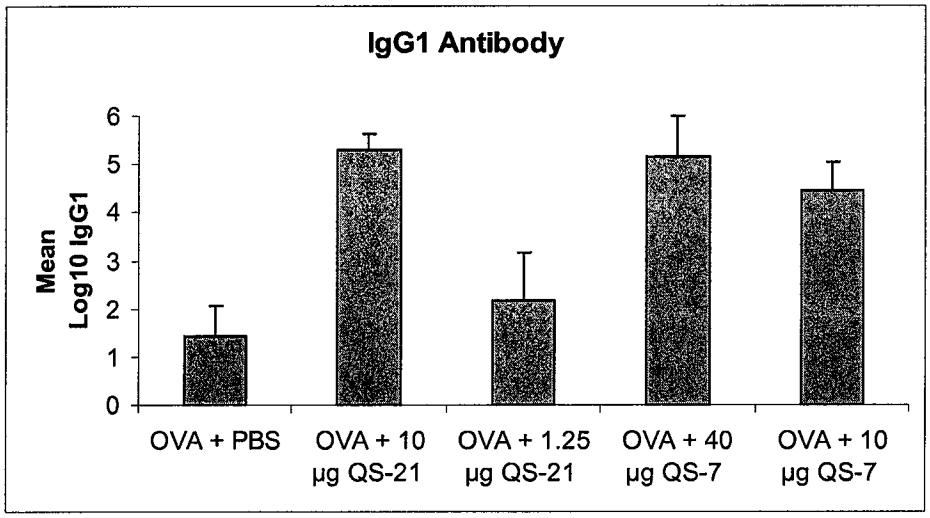
SLIDE C
Interferon-gamma ELISPOT



ANTIGENICS

EXHIBIT D
Page 4 of 8

SLIDE D
Anti-OVA IgG1



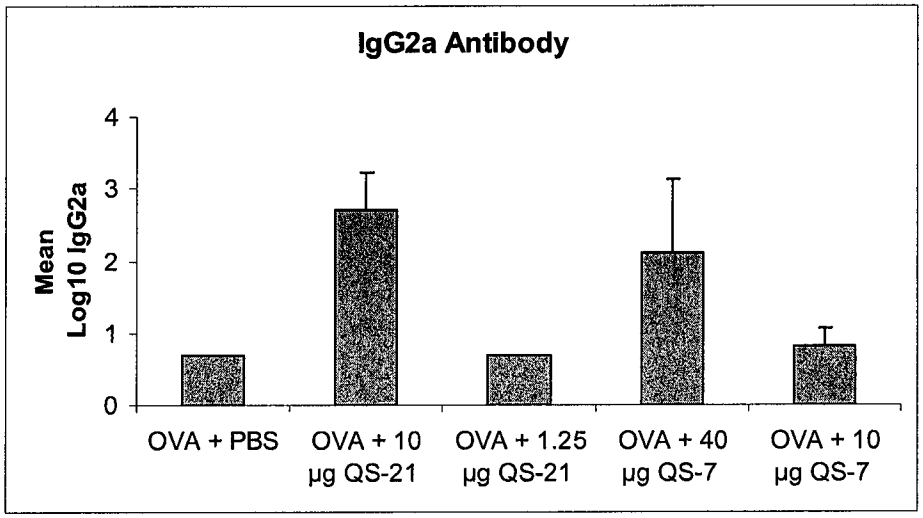
*Significant at $p \leq 0.05$ by Dunnet's test vs OVA + PBS control.

Error bars show sd for individual mice.

ANTIGENICS

EXHIBIT D
Page 5 of 8

SLIDE E
Anti-OVA IgG2a



*Significant at $p \leq 0.05$ by Dunnet's test vs OVA + PBS control.

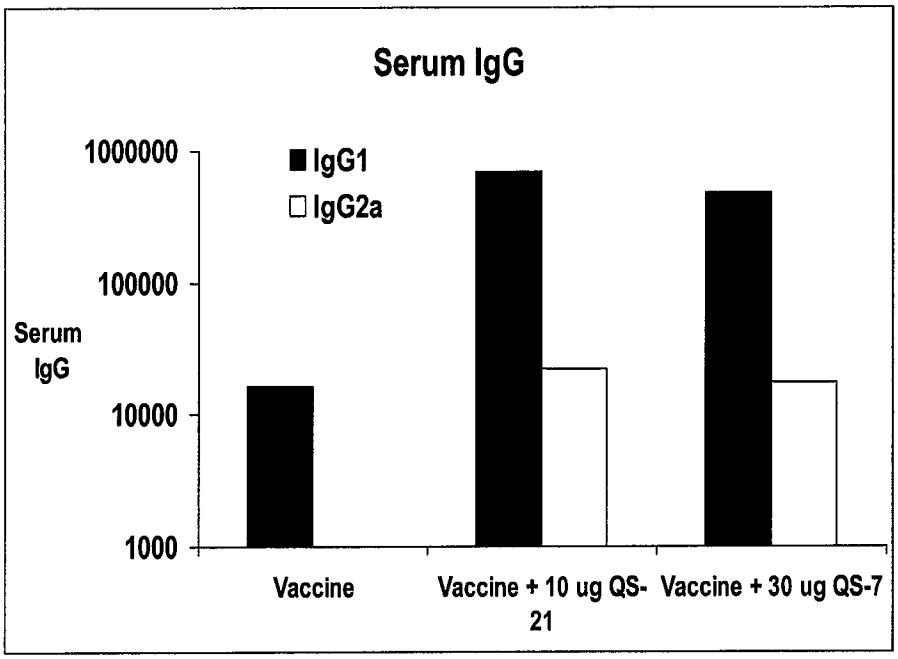
Error bars show sd for individual mice.

ANTIGENICS

EXHIBIT D
Page 6 of 8

SLIDE F

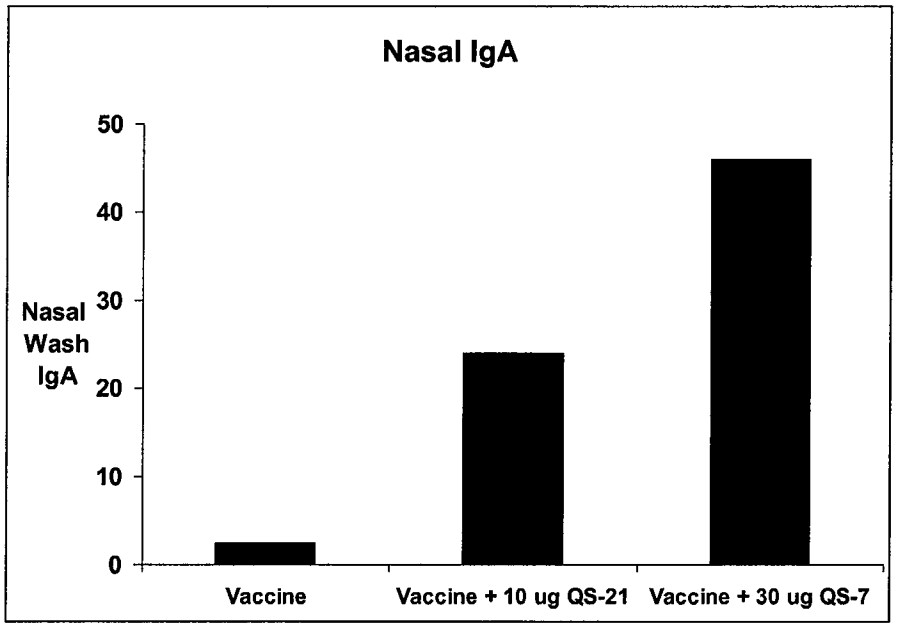
Flu Vaccine – Serum IgG after Intranasal Imm.



ANTIGENICS

SLIDE G

Flu Vaccine – Nasal IgA after Intranasal Imm.

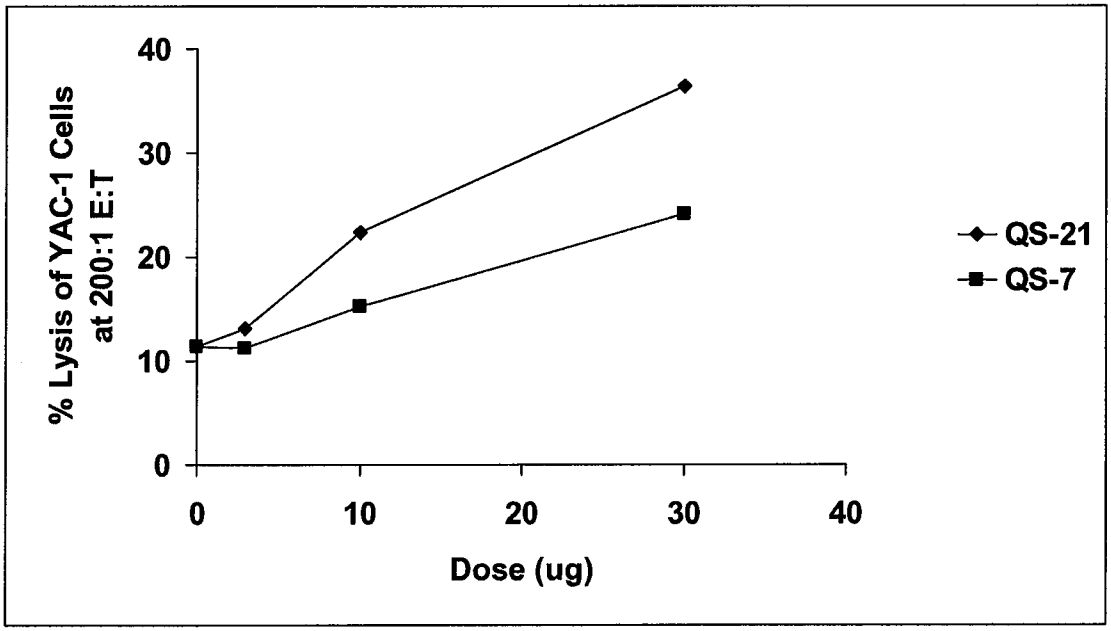


ANTIGENICS

EXHIBIT D
Page 8 of 8

SLIDE H

Dose Response for Induction of NK Activity



BALB/c mice (5/group) were administered formulations containing the indicated doses of QS-21 or QS-7 on days 1 & 2. On day 3, splenocytes were assayed for NK activity by a standard ^{51}Cr release CTL assay against the NK-sensitive YAC-1 lymphoma line. Splenocytes were assayed as a pool for each group.

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